Comparative Assessment of Bioavailability and Toxicity of Zinc Nanoparticles in Animal Model

M.Phil. Thesis

By

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Comparative Assessment of Bioavailability and Toxicity of Zinc Nanoparticles in Animal Model

Thesis Submitted by

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DECLARATION

I hereby declare that the thesis entitled **"Comparative Assessment of Bioavailability and Toxicity of Zinc Nanoparticles in Animal Model"** submitted at Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad for the award of degree of Master of Philosophy in Pharmacy (Pharmacology) is the result of research work carried out by me under the supervision of my supervisor Dr. Muhammad Khalid Tipu during the period 2017-2019. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship and I am aware of the terms copyright and plagiarism and I will be responsible for any copyright violation found in this work.

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This is certified that the dissertation titled "**Comparative Assessment of Bioavailability and Toxicity of Zinc Nanoparticles in Animal Model**" submitted by **Mr. Muhammad Qasim** to the Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan is accepted in its present form as it is satisfying the dissertation requirements for the degree of Master of Philosophy in **Pharmacy (Pharmacology).**

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No two things have been combined together better than knowledge and patience" "Mohammad (PBUH)" I dedicate my work to ALLAH ALMIGHTY who is the most merciful and helpful and my precious Mother and Father, whose affection, love, encouragement and prayers of day and night make me able to get success and pride. And some part of thesis is dedicated to caffeine and sugar as well, my companions through many a long night of writing

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Muhammad Qasim

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List of Abbreviation

Abbreviation	**Description**
AUC	Area under the Curve
Ca	Calcium
CDF	Cation diffusion facilitator
Cl	Clearance
Cmax	Maximum plasma concentration
Cu	Copper
DNA	Deoxyribonucleic acid
Fe^{+3}	Ferric
GIT	Gastrointestinal track
Hr	Hour
Kg	kilogram
Mg	Magnesium
mg	Milligram
Mn	Manganese
MRT	Mean residence time
MT	Metallothionine
NIH	National institute of health
nm	Nanometer
Pdi	ploy dispersity index
RPM	Revolution per minute
SLC30	Solute carrier 30
t_{max}	time to reach C_{max}
TPP	Tripolyphosphate
Vit-B6	Vitamin B 6
ZIP	Zrt-like-protein
Zn	Zinc

ZnN	Zinc Nanoparticles
ZnP	Zinc Plain
Znt	Zinc transporter

Abstract

Zinc is an important micronutrient having multiple biological roles. Being integral components of more than 300 enzymes of all six classes and various transcription factors, it plays vital role in cellular signaling and functioning especially in immune cells and hepatocytes. Zinc in various form have been used for various pathological conditions like Wilson's disease, common cold, hepatic malfunctions and acrodermatitis enteropathica. The main problem in zinc supplementation/therapy is its erratic bioavailability and pharmacokinetics. The main site for zinc absorption is jejunum. Various cellular zinc transporters play a significant role in zinc absorption. These transporters proteins are greatly affected by other dietary divalent cations e.g. calcium, iron, and magnesium. Furthermore, administrations of higher amount of zinc results in the saturation of these transporters. Nanotechnology has been applied in pharmaceutical sciences to enhance the bioavailability and cellular uptake of various drugs. Zinc oxide nanoparticles were developed to overcome this problem, but these formulations were linked to oxidative stress in the experimental animals. To address the problem efficiently, current study was designed to prepare zinc sulfate nanoparticles to avoid oxidative stress with enhanced bioavailability. Zinc nanoparticles were prepared using zinc sulfate nanoparticles by inotropic gelation method and characterized using scanning electron microscope (SEM) and zetasizer.

Sprague Dawley Rats (n=27) were equally divided into three groups receiving: zinc sulfate nanoparticles (4 mg/Kg); plain zinc sulfate (4 mg/Kg) and normal saline to keep in account the diurnal variation. All the drugs were administered orally. Blood samples (0.3 to 0.5 ml) were collected randomly from tail vein at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 72 and 120 hours from three animals in each group. After separating the plasma, samples were processed and analyzed for concentration of zinc by atomic absorption spectroscopy. Plasma zinc concentrations thus obtained were analyzed by one-way analysis of variance (ANOVA) with Duncan test for multiple comparisons. The plasma concentration vs time curve was used to calculate half-life, C_{max}, t_{max}, AUC (0 to t), clearance and mean residual time by PK solver software using non-compartment model. For safety/toxicity studies albino mice (n=54) were randomly segregated into six groups: group I received normal saline (control); group II received plain zinc sulfate (40 mg/kg); group III received zinc oxide nanoparticles (40 mg/kg). while group IV, V and VI received 2 mg/kg, 4 mg/kg and 40 mg/kg of zinc sulfate

nanoparticles respectively through gavage daily for 21 days. Food and water were provided ad libitum. Blood sample (approximately 1 ml) was collected from three animals in each group weekly for hematological examination and clinical chemistry. Liver, kidney and lungs were isolated form the decapitated animals and processed for histopathological examination.

Results of study showed significant rise in plasma zinc concentration in animal receiving zinc sulfate nanoparticles than that of plain zinc sulfate after 1 hour of administration of zinc nanoparticles ($p<0.05$). The increase was remained significant till the end of the study (120 hours). Furthermore, significant rise in C_{max}, AUC_{0-t} and mean residual time were recorded in animal receiving the zinc nanoparticles. Moreover, data of studies showed improvement in bioavailability and pharmacokinetics parameters of zinc in nanoparticles formulation as compared to conventional zinc preparation. Animals given zinc oxide nanoparticles indicated significant rise in bilirubin, ALT, AST, ALP and creatinine level than that of animals received normal saline (group I). Whereas, significant decline was observed in serum total protein level was observed in these animals than animals of the group I. Moreover, sever degenerative changes in liver, kidney and lungs were present in these animals. However, animals receiving zinc nanoparticles didn't indicate such degenerative changes. The Serum and hematological profile of these animals are comparable with animals receiving plain zinc sulfate. It is concluded from findings of the study that orally administered zinc sulfate nanoparticles has significantly increased the bioavailability of zinc and appear to be less toxic as compared to zinc oxide nanoparticles.

CHAPTER 1

INTRODUCTION

1. 1. INTRODUCTION

1.1. Physical Property of Zinc

Zinc, a metallic element of bluish white color has vast industrial application. The symbolic representation of the zinc is Zn, having atomic number of 30 which is present in the group II B in the periodic table. German scientist Andres Sigismund Marggraf produced the pure zinc in 1746 by heating the calamine and charcoal (Kropschot and Doebrich, 2011).

1.2. Zinc Content in the Human Tissue

The total content of zinc in body is about to 2-3 grams (Figure 1.1). Skeletal muscles contain about to 60% while 30% of the zinc is stored in the bone. The liver as well as the skin contain the 5% of zinc and the remaining body tissues contain 2- 3% of the zinc (Roohani *et al.*, 2013). Plasma contains a very small amount of zinc (0.1%). Out of the small amount, 80% is loosely bound to albumin while 20% is very tightly bound to 2-macroglobolin (Barnett *et al.*, 2013; Reyes, 1996). Human have the ability to regulate the total body zinc content even when there is a 10-fold decrease in the intake (Fukunaka *et al.*, 2011; Kambe *et al.*, 2015).

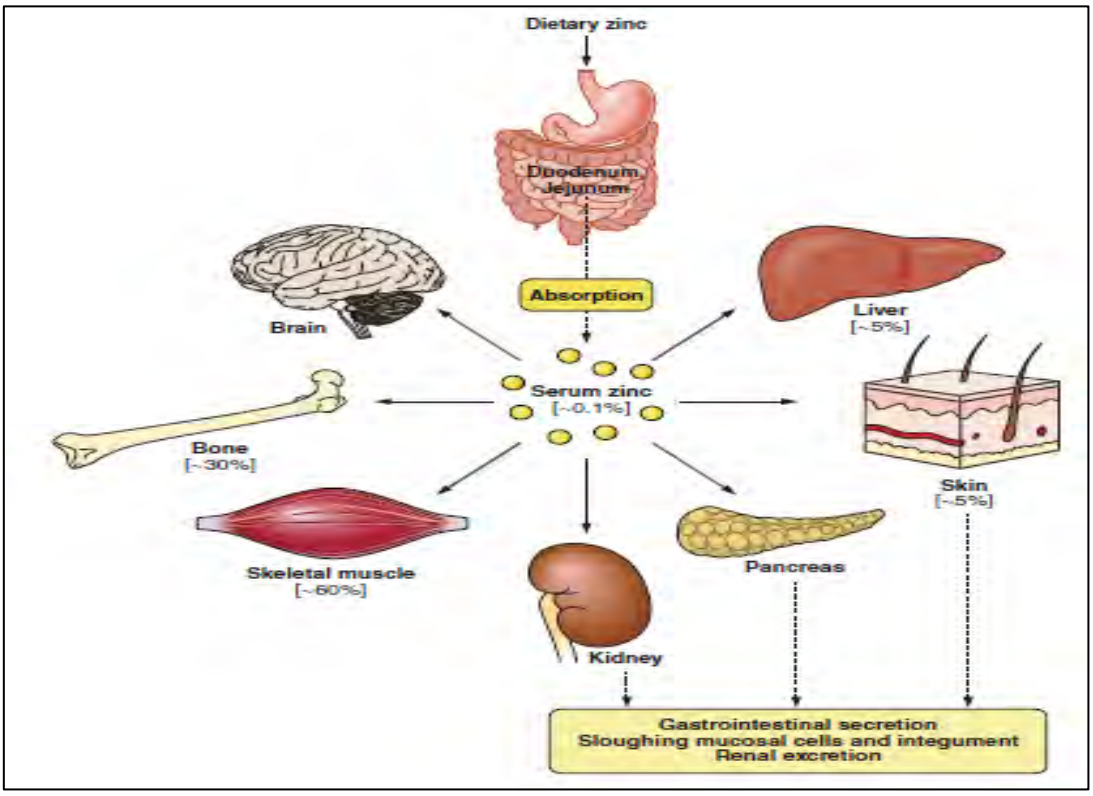

Figure 1.1. Distribution of zinc in human body

1.3. Pharmacodynamics of Zinc

Zinc is an integral part of more than 300 enzymes and transcription factors, it perform various important biological function by following mechanisms:

1.3.1. Structural mechanism

Physio-chemical characteristics of the zinc play a vital role in the structural function of the zinc. There are many proteins which take part in the DNA replications and reverse transcription, Zinc also serves as structural and functions component for other proteins for example ceruloplasmin and carbonic anhydrase. Metalloproteins functions may be badly affected due to this function of the zinc (Mocchegiani *et al.*, 2000). Zinc is necessary for the structural integrity of the chromatin and cell membranes and for the function and synthesis of hormones (Tapiero and Tew, 2003).

1.3.2. Catalytic mechanism

Catalytic role of the zinc is very important as enzymes involved in the catalysis and co-catalysis. Zinc takes part in biological processes as a catalytic agent like in synthesis of DNA, growths, development of the brain, reproduction, behaviors, fetal development, stability of the membrane, formation of the bone and wound healing and immune cells (Mocchegiani *et al.*, 2006).

1.4. Clinical Pharmacology of Zinc

1.4.1. Therapeutic uses of the zinc

By virtues of its diverse physiological roles deficiency of zinc causes following clinical manifestation:

i. Growth retardation: zinc deficiency results in the delayed growth in the growing children,
ii. Hypogonadism: zinc deficiency in men also leads to a gonadal dysfunction.
iii. The most importantly deficiency of the zinc causes impaired perception and cognitive functions especially in children.
iv. Another most important complication of the Zinc deficiency affecting children and infants is acute diarrhea in the under developed countries (Prasad, 2012).

v. Zinc is reported to decrease the severity of common cold (Singh and Das, 2013).
vi. FDA approved zinc for the treatment of Wilson's disease a hereditary disorder which is produced due to copper accumulation. When the liver copper is excreted in the bile it may produce defect in the bile circulation. When the excessive amount of copper accumulated in the liver, brain and kidney, it leads towards the toxicity. It can be prevented by zinc (Brewer, 1995).
vii. Zinc treatment/therapy decreases the symptoms and mortality rate in patients suffering from macular degeneration (Clemons *et al*, 2004).

1.4.2. Side effects of zinc therapy

Many studies were conducted by the researchers to documents and to investigate zinc deficiency. But attention and care must be given to the adverse effects of the zinc supplementation. The excessive intake of zinc my produce some unwanted effects as zinc nutritional supplement so considered as non-toxic relatively when takes orally however indication of over toxicity may causes the following symptoms.

i. Nausea
ii. Vomiting
iii. Lethargy
iv. Epigastric pain
v. Fatigue

These symptoms will appear when there is high intake of zinc occurs. The daily recommended allowance of zinc is 100-300 mg Zn/day vs. an recommended daily allowance of 15 mg Zn/d .When the recommended daily allowance is exceeded then the symptoms of copper deficiency appears like anemia :microcytic hypochromic, with increased erythrocyte fragility leading to hemolytic anemia (Fox, 1989) The other symptoms include neutropenia, decreased in immune function. Moreover, ratio between low density protein and high density protein is affected (Fosmire, 1990, Maret and Sandstead, 2006).

1.4.3. Toxic effects

Beside the beneficial effect of the zinc sulfate which is used in many pathological condition (previously described). Some toxic effects are also associated with the zinc

sulfate. A study reported that zinc sulfate was safe up to 300 mg orally for two weeks. Zinc produces a toxicity signs in higher doses. Growth retardation, abnormalities in the hematological parameters, deteriorating changing in the pancreatic gland and significant changes in the biochemical parameters were reported in study (Maita *et al.*, 1981) Some histopathological changes were also reported as toxic lesions in the stomach, intestine, spleen and kidney of the mice and rats (Maita *et al*., 1981).

1.5. Pharmacokinetic of Zinc

Nano formulations of zinc increase the plasma concentration by enhancing the absorption as compare to plain zinc. Previously many studies conducted showed that zinc oxide at a dose of 2.5 g/kg achieves maximum plasma concentration after 6 hours of administration (Zhang *et al.*, 2018a). Another study indicated that maximum concentration was achieved after 2 hours, where gradual increase was observed after 30 minutes of the administration (Li *et al.*, 2012).

1.5.1. Absorption

All part of small intestine is responsible for absorption of the zinc but most of the absorption of the zinc occurs in the duodenum and jejunum. There is a 90% increase increases in absorption in case of declined dietary zinc intake (Taylor *et al.*, 1991). When there is rise in dietary zinc level, zinc is eliminated from the body through integumentary system and renal excretion (Hambidge and Krebs, 2001; Krebs, 2013). 20-40% of the dietary zinc is absorbed on daily basis from the enterocytes. Absorption of the zinc salts is much better in empty stomach than with the food. Dietary contents especially phytate and divalent cations (Cu^{+2}, Mg^{+2}, Ca^{+2} & Fe^{+2} may affect the bioavailability of zinc (Lonnerdal, 2000).

1.5.2. Distribution

When absorption of the zinc is completed then zinc transportation occurs via portal circulation to the liver. Hepatocytes retain some amount of the zinc while systemic circulation is responsible for remaining amount of the zinc to be distributed. Zinc may be bound to various plasma proteins by different affinities. Binding affinities of the zinc for plasma protein is as, (Scott and Bredwell 1983).

1. Albumin 70-80% loosely bound
2. Alpha-2 microalbumin 10-20% tightly bound
3. Transferrin 5-10%

1.5.3. Intracellular distribution of zinc:

Uptake of the zinc into the cell occurs within the minutes when zinc is added into the cell culture. (Reyes 1996; Wellinghausen 1996). Zinc transporters are responsible for the transportation of zinc across the cell and such transporter (ZIP) is proteinous in nature and were first identified in the nervous system (Palmiter and Findley 1995 In the enterocytes initial 30 minutes of the zinc uptake occur very rapidly after which saturation occur within 30 -60 minutes (Willinghausen, 1996). Cytoplasm contains 50% while nucleus and cell membrane contain 40 and 10% of zinc respectively (Colvin *et al.*, 2008; Krezel and Maret, 2006). As zinc is mainly present in bounded form so the freely available zinc is very low in the cytosol which is present in the range of picomolar and nanomolar.(Kambe *et al.*, 2015a; Outten and O'halloran, 2001, Qin *et al.*, 2011).

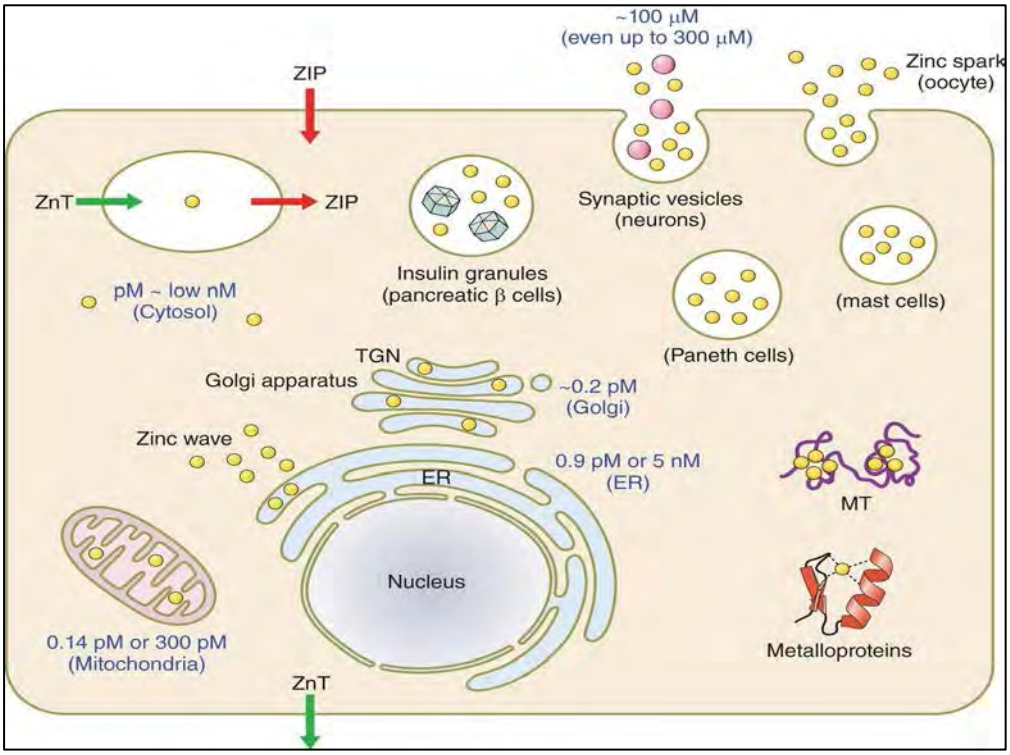

Figure 1.2. Cellular distribution of zinc

There is evidence that in response to the various stimuli the zinc amount may be altered which is shown by the zinc wave above. Zinc concentration in various intracellular organelles is shown in Figure 1.2. The amount of that free zinc concentration is measured about 0.14 pM in the mitochondrion (Park *et al.*, 2012). While 0.2 pM is present in the mitochondrion matrix (Qin *et al*., 2011), Endoplasmic reticulum and Golgi bodies contain 0.9 pM & 0.2 pM of zinc respectively. The much

higher concentration is present in the mitochondrion and endoplasmic reticulum (Maret, 2013).

1.5.4. Excretion of the zinc

Mainly 27-90 mmol/day zinc is eliminated from the intestine through feces while 8-11 mmol/day zinc is eliminated in the urine. Urinary excretion of the zinc is low as compared to the fecal excretion because of reabsorption taking place in the kidney. Another site which is responsible for the zinc excretion is integumentary system such as epithelial cells, hairs and sweat. Some amount of zinc is also excreted in semen (Medicine, 2001).

1.6. Zinc Transporters

Zinc transportation into the cell is regulated through the following mechanisms:

1: Non-saturable, non-mediated mechanism.

2: Saturable-carrier mediated mechanism.

There are two types of transporters proteins for zinc movement across the cell. These are encoded by two separate genes families.

1.6.1. ZIP family zinc transporters:

ZIP (Zrt-Irt-like protein), the name is given due to the presences of Zrt1 protein in yeast. Zrt1 and Zrt2 are the primary zinc uptake transporters in the yeast *(Saccharomyces cerevisiae)* ZIP is the member of this family which is firstly identified. SLC39 is the systematic designation which is given to the mammalian members of this family (Pflugers Arch 2004). The basic role of the ZIP family proteins is uptake zinc from extracellular environment to cytoplasm. The same is also responsible for the uptake of other divalent cations like Ca^{+2}, Mg^{+2} & Fe^{+2}. Such transporters are found in bacteria, fungi, plants and mammalian cells (Gaither and Eide, 2001).

The recently discovered mammalian zinc transporters are very important to be focused. There are 14 sub family of ZIP and all these 14 family members are encoded by the human genome (Eide, 2004). Out of these 14 ZIP transporters, ZIP1/slc39a1 is present in the mice and human (Dufner-Beattie *et al.*, 2003a, Gaither and Eide, 2001b). The role of such proteins is to take part in the uptake of zinc for example ZIP1 transporters take part in the uptake of zinc in red blood cell, leukemic cells and prostate cells (Costello *et al.*, 1999, Franklin *et al.*, 2003).

ZIP1 is mainly not present on the plasma membrane of all the cells under normal conditions but deficiency of zinc results in expression of some of these proteins for zinc homeostasis. Usually these protein are subcellular localized to the plasma membrane (Gaither and Eide, 2001b, Milon *et al.*, 2001).When there is deficiency of zinc in the cell so there is migration of the ZIP4 into the plasma membrane (Wang *et al.*, 2004). In mammalian cells ZIP2/slc39a2 and ZIP3/slc39A3 proteins are present, which are responsible for the uptake of zinc (Dufner-Beattie *et al.*, 2005; Dufner-Beattie *et al.*, 2003b; Kelleher and Lonnerdal, 2005). The important transporter which takes part in the uptake of zinc from the diet is ZIP4/SLC39A4 protein present on enterocytes and responsible for uptake of dietary zinc. The dietary absorption of the zinc which is carried out by this protein first explored by the two research groups who were studying mutation in the human gene which control the genetic disorder acrodermatitis enteropathica (Wang *et al.*, 2002). The Zip transporters are mainly limited into the cell surfaces but one of the transporters ZIP5 which are not present in the cell surface, but deficiency of zinc causes the expression the ZIP5. There are eight type of transmembrane domains of the ZIP proteins which is mainly present on the plasma membrane as shown in Figure 1.3 (Eide, 2006; Gitan *et al.*, 2003).

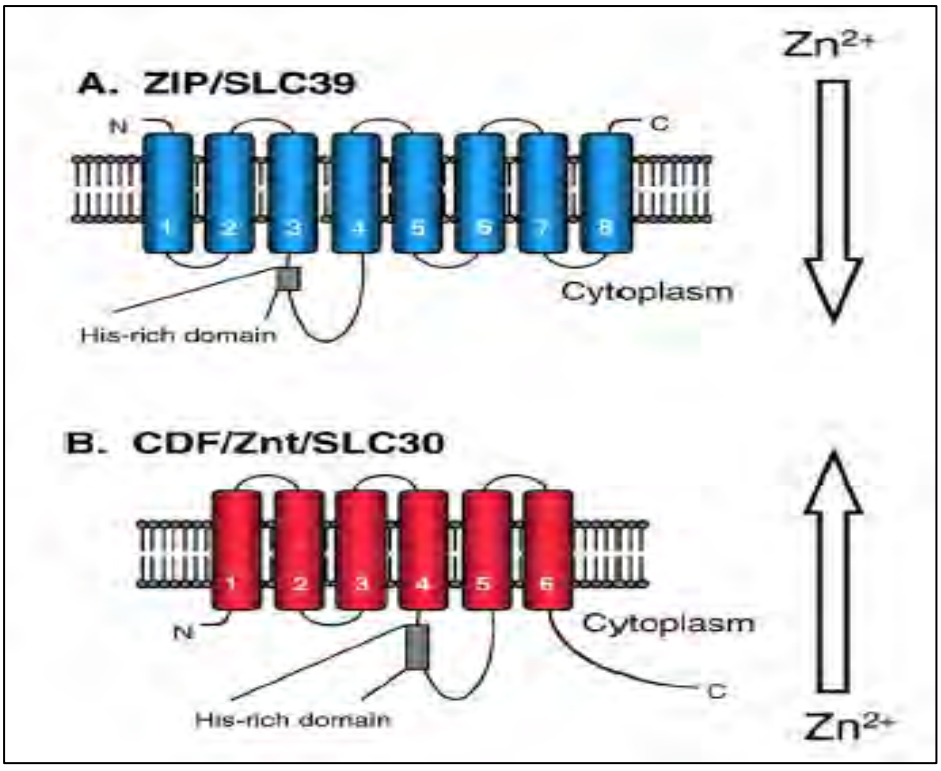

Figure 1.3. ZIP and ZnT family transporters

1.6.2. Znt family zinc transporters

Zinc transporters (Znt family) is also known as CDF which is stands for the cation diffusion facilitator (Nies and Silver, 1995). SLC30 is the Systematic name which is given to the mammalian member (Znt) (Gaither and Eide, 2001a). Znt family is responsible for the exocytosis of zinc and other metals ions so the said process is opposite in direction to that of Zip family transporters. There are six projected transmembrane domains of most of the member of this family as shown in Figure 1.3. Znt transporters have the same motif as like the ZIP transporters which are histidine rich in nature. The transmembrane domains are usually present in the cytoplasmic loop between 4 and 5 (Figure 1.3) and during transportation of the zinc these domains binds to the metals i.e. these domains are responsible for the metal binding (Persans *et al.*, 2001).

Generally, the CDF transporter are divided into the following three groups (Montanini *et al.*, 2007):

i. Zn-CDF
ii. Zn/Fe-CDF
iii. Mn-CDF

The distribution of ZIP and ZnT family transporters proteins is illustrated in Figure 1.4 (Kambe *et al.*, 2015)

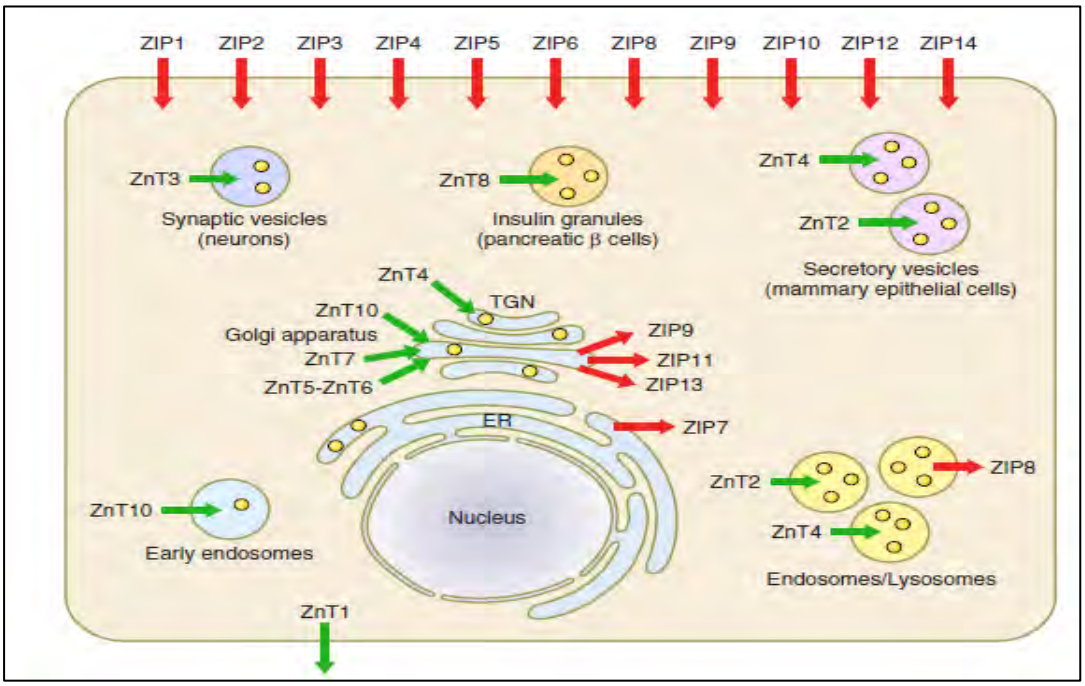

Figure 1.4. Locations and functions of zinc transporters

1.7. Factors Affecting Zinc Absorption

Zinc absorption from the enterocytes occurs mainly through saturable active transport as compared to non-saturable passive transport (Steel and Cousins, 1985). High concentration of the Zinc is absorbed form the intestine through active transport (saturable process). While non-saturable process may take part in absorption of zinc which is concentration dependent pathway. This pathway depends upon the cation's concentration. So, there are many factors/dietary components which affect absorption of the zinc from the intestine are as follow.

1.7.1. Compounds increasing the absorption

Zinc absorption is enhanced in the presence of some compounds like picolinic acid which is secreted by the pancreas. Another compound which increases absorption of the zinc is vitamin –B6 (Pyridoxine) which stimulate the picolinic acid secretion from the pancreas. Citrate and amino acid may contribute to increase the absorption of the zinc. Amino acid like histidine , lysine and glycine, cysteine and methionine enhance the absorption of the zinc (Salgueiro *et al.*, 2000a). Daily Protein intake in the diet may increases the zinc concentration in GIT so considering these proteins are the main sources of the dietary zinc, these sources are not only taking part to increases the presence of the zinc, but it also responsible for the availability of the zinc in the diet(Sandstrom *et al.*, 1980). This is due to the digestions of the proteins which are metabolized and form intermediate products which bind with the zinc and help in the absorption of the zinc. These all changes are dependent on the digestion of the proteins, chemical composition of the peptides and in turn amino acids content of the proteins, for example Sulphur containing amino acids like cysteine (Lonnerdal, 2000).

1.7.2. Compounds decreasing the absorptions

Some compounds are responsible for their inhibitory action on the absorption of zinc. Like calcium (Wood, 2000), iron (Solomons and Jacob, 1981), selenium (House and Welch, 1989), folic acid (Ghishan *et al.*, 1986), phytic acid (Cichy *et al.*, 2005), tannins and dietary Fibers (Salgueiro *et al.*, 2000b).

Different types of divalent cation transporters are present at the brush border membrane of intestine (Gunshin *et al.*, 1997). The same transporters are used for the transport of zinc. Various multivalent cations can compete with each other for the transporter and can decrease the movement of other ions subsequently According to

one of the study effects of different cation like Mg^{+2}, Cu^{+2} and Mn^{+2} decrease the zinc absorption by competitive antagonism at common transport proteins. The inhibitory effect of these cations depend upon the concentration and the affinity of these cation for the metallic transport protein (Bertolo *et al.*, 2001). Saccharides can be used to overcome this inhibitory effect. Phytic acid is abundant in the legumes, fruits and vegetables. It has charged phosphate groups (6) having greater binding capacity for the cations like zinc, calcium in the GIT. It makes complex with zinc and reduces its absorption. Phytic acid is also reasonable for the interaction of zinc and calcium. Phytic acid is also reasonable for the interaction of zinc and calcium. Both the nutritional supplements are the antiport of each other (Lopez *et al.*, 2000).

1.7.3. Metallothionine *(*MT)

Metallothionines are abundant proteins which are categorized mainly by low molecular weight, without aromatic amino acid and with high content of cysteine (West *et al.*, 1990). Metallothionines occur in the animals, bacteria, plants and fungi. The human metallothionine family is encoded by at least 17 related genes. Metallothionines mainly bound to zinc, cadmium and copper (Bremner, 1991).Metallothioneins/thioneins are the important components of cellular zinc homeostasis. When there is increase in the concentration of zinc abruptly so these metallothionines/thionines control the zinc concentration by making complex with cellular zinc. While when there is decrease in the zinc concentration metallothionines gives up the zinc and changes into thionins, The metallothioneins and thionines keep the zinc concentration fairly constant (Krężel and Maret, 2017).

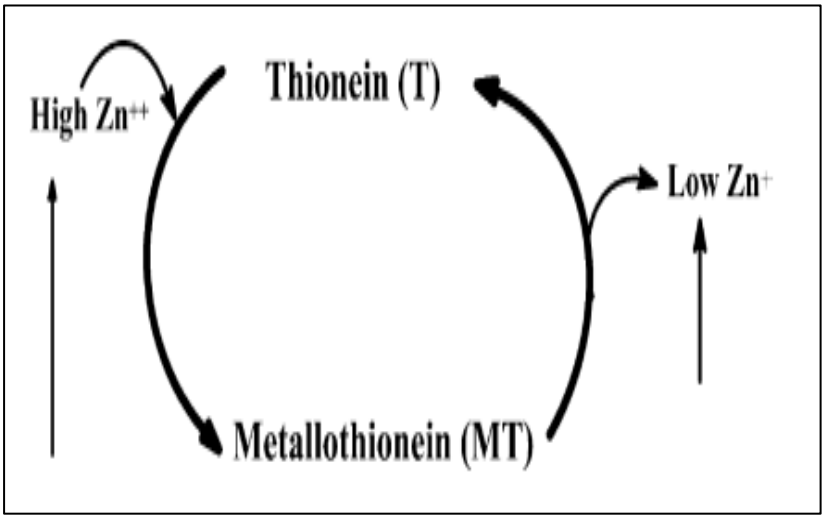

Figure 1.5. Homeostatic control of zinc by metallothionines and thionins

1.8. Methods to Improve the Bioavailability of Zinc

As zinc bioavailability is affected by many factors in which the most important factor is phytic acid concentration in the food. A decrease in phytic acid concentration may boost up the zinc absorption from GIT. Second major factor which is responsible for the low absorption of the zinc is antiport or antagonist of zinc. The antagonists are mainly divalent cations like calcium, copper and iron etc. which completely bind to the same transporters of the zinc. Saccharides can be used to inhibit the antagonistic effects of divalent cations as discussed previously.

1.8.1. Nano technology

Nanoparticles are mainly referring as the small materials having size less than 1000 nm along with the specific physiochemical properties and the said properties are different from the parent materials which have the same composition (Medina *et al.*, 2007). Nanoparticles show their action at cellular level and produce desirable effects at low doses. But nanoparticles can produce some unwanted effects too. When medicine is used as a nanoparticle for therapeutic effects, it must be evaluated for the undesirable effects of the nanoparticles. Therefore, careful attention must be given to a side effects and benefits of the nanoparticles which are used as nanomedicine. Nanoparticles because of their characteristics properties have beneficial impact and role in the field of pharmaceutical and medical science (Medina *et al*., 2007).

1.8.2. Nanoparticle as a drug delivery system

The classical strategies used for the development of the pharmacological agents is being limited either due to the pharmacokinetics or pharmacodynamics problem like having low efficacy and non-selective mode of action, Moreover non-effectiveness of the drug is the main problem at the target site due to the physiological barriers or cellular mechanism. Furthermore, low bioavailability, low solubility and shorter half-life are the factors affecting the therapeutic outcome. The solution to overcome such problems is particle size reduction (Nano range). Particles in the Nano range can easily cross the physiological barriers and can enter into the target cells. Nanoparticles are prepared mainly for the entrapment purposes, encapsulation purposes, more stability, solubility and to increase the absorption of the drugs. Another purpose of the Nano formulation is to control the drug release from the formulation. The modern technique may protect the drug form premature/early inactivation when the drug is

transported in the blood. Through Nano technology we can transport different type of therapeutic agents like DNA, peptides, proteins and low molecular weight compounds too. Nano particles are used as a drug delivery system in which the most popular and beneficial are liposomes and polymers-based nanoparticles. All these complexes are biodegradables and are not going to accumulate in the body. That results in minimum risk to the living cells (Sapra *et al.,* 2005). However, there is a need to investigate the drug release and the toxicological profile of the nanoparticle (Foster and Hirst, 2005).

1.8.3. Use of nanotechnology for zinc

Zinc oxide nanoparticles are one of the most commonly used because zinc oxide nanoparticles have unique physiochemical properties. Zinc oxide nanoparticles are used as antibacterial, catalytic, ingredient in ceramics, cosmetics, paints and in food industries as additives. In agriculture it is used as fungicides. While in the field of health sciences it is reported to possess anticancer activity (Ye *et al.*, 2016). Furthermore It is also used in animals feed as micronutrients (Jacobsen *et al.*, 2015).

1.8.4. Toxicity of zinc oxide nanoparticles

Along with the beneficial use of zinc oxide nanoparticle, the toxic and unwanted effects of these particles have been reported. These particles adversary affect the circulatory (vascular obstruction), pulmonary, immune cells and digestive system (Attia *et al.*, 2018; Kaya *et al.*, 2016). Many studies showed that zinc oxide nanoparticles produce toxic effect i.e. membrane damage, inflammation, DNA damage, changes in hormones level and apoptosis (Patil *et al.*, 2016; Zhang *et al.*, 2017). These damaging effects are linked with production of oxidizing radical and oxidative stress. As shown in the Figure 1.6

1.8.5. Chitosan based zinc sulfate nanoparticles

Chitosan is biodegradable biocompatible and nontoxic. Chitosan is used to increase the absorption of orally administered drugs. As chitosan has the mucoadhesive properties and ability to open tight junction in the mucosal cells membrane. These two properties are responsible for absorption promoting effect of chitosan (Sadzuka *et al.*, 1998). Chitosan is positively charge while mucin is negative charge so the interactions between these two charges results in prolong contact time between the adsorptive surface and drug. It also results in control release of the drug / active ingredient from the polymer (Aspden *et al.*, 1996; Vila *et al.*, 2002a).

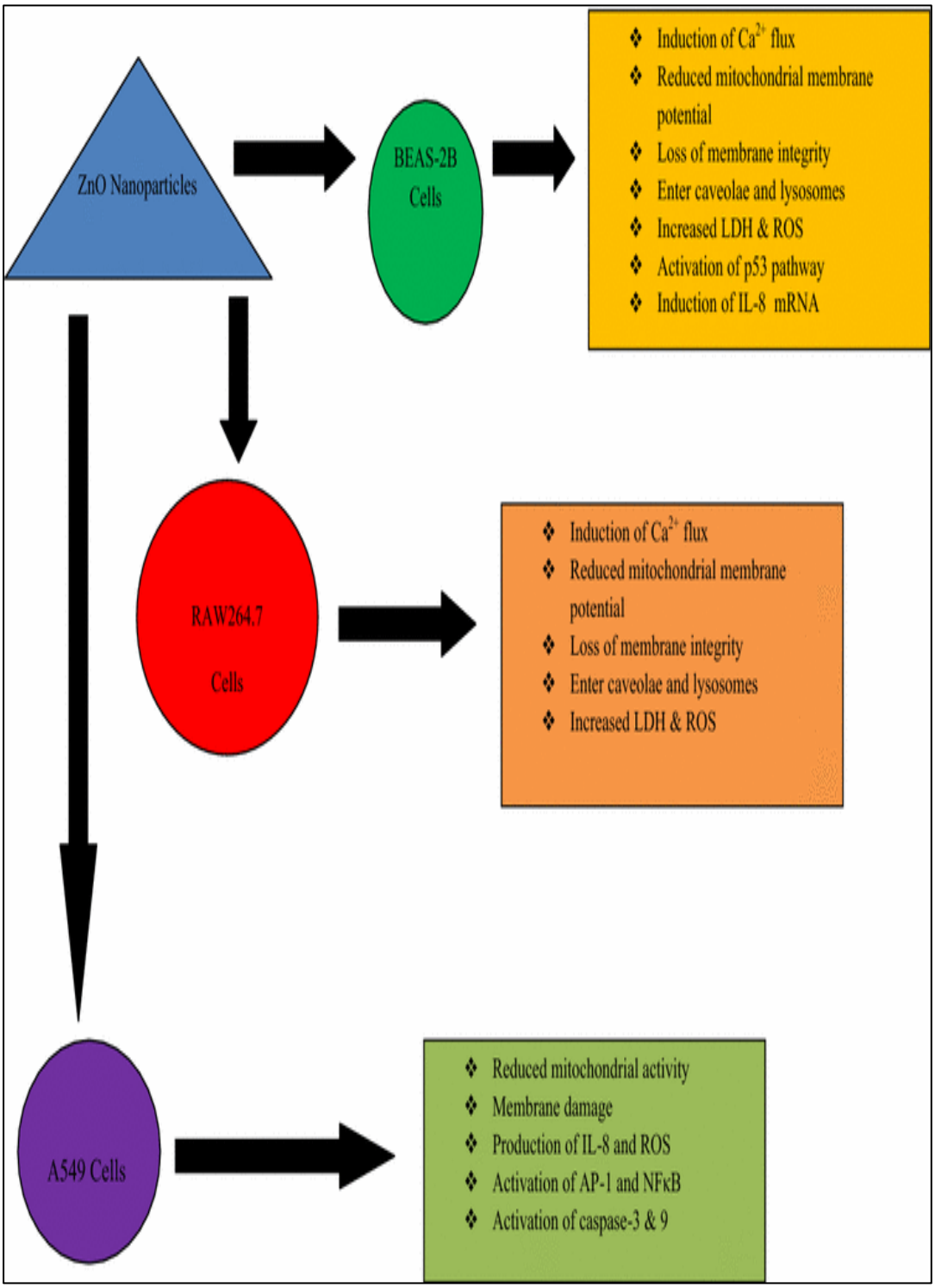

Figure 1.6. In vitro toxicity of zinc oxide nanoparticles

In order to control the saturation mechanism of the zinc transporters protein and to increases the bioavailability of zinc, chitosan-based zinc sulfate nanoparticles were formulated to enhance the absorption & decrease the chance of oxidative stress (Zhang *et al.*, 2018).

1.9. Aim and objectives

The aim of study was to evaluate and compare bioavailability and safety of chitosan-based zinc sulfate nanoparticle with following objectives:

1.9.1. Objectives

- Preparation and characterization of Zinc sulfate nanoparticles.
- Measuring the bioavailability and pharmacokinetic profile of the Zn nanoparticles and comparison with plain zinc sulfate formulation.
- To assess and compare the toxicity of Zinc nanoparticles with plain zinc sulfate and zinc oxide nanoparticles.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Chemicals

Low molecular weight chitosan (C3646), zinc sulfate heptahydrate (Z0501 Sigma), sodium tripolyphosphate (TPP) (N238503), sodium hydroxide (221465-500G), potassium phosphate monobasic (P8709 Sigma), were purchased form Sigma Aldrich, Germany. Glacial acetic acid, analytical grade (AC03521000) was purchased from Scharlab, Spain. Nitric acid analytical grade (UN 2031) was purchased from Honeywell Riedel-de haen, Germany. Zincat® from Atco Laboratories, Karachi, Pakistan was purchased from well-reputed local pharmacy.

2.2. Preparation of Chitosan Nanoparticles

Ionotropic gelation method by Du et al (2009) was used for the preparation of chitosan-based nanoparticles with slight modification in the concentration of the tripolyphosphate solution, concentration of the chitosan solution and speed & timing of centrifugation. Briefly, 0.2% (w/v) chitosan solution was prepared by dissolving 50 mg chitosan in 25 ml of 1% (v/v) glacial acetic acid. TPP solution was prepared by dissolving 10 mg in 10 ml of distilled water in order to obtained 1% TPP solution. Tripolyphosphate solution were added to the chitosan solution under magnetic stirring at 800-1000 RPM using Hotplate magnetic stirrer (MSHP 1A111, PCSIR, Islamabad, Pakistan) at room temperature. The mixture was allowed to stir for 30 minutes, and then it was sonicated at 1.5 kW for 20 minutes. Afterward, the solution was centrifuged for 15 minutes at 12000 RPM. A colorless pellet was obtained on the side of the tube, and it was rinsed with distilled water. Finally, the obtained pellet was suspended in distilled water.

2.3. Loading of zinc into the nanoparticles

Chitosan (40 mg) was dissolved in 25 ml of 1% acetic acid solution. Five milliliters zinc sulfate solution 25% w/v was added to the chitosan solution under magnetic stirring followed by the addition of TPP solution to the mixture. The mixture was stirred continuously for 12 hours. Afterword the solution was sonicated for 20 minutes. The solution was centrifuge for 20 minutes at the 12000 RPM. A whitish pellet was obtained after the removal of the supernatant and washing with distilled water. Supernatant was used to determine encapsulation efficiency. Finally pellet of nanoparticles loaded with zinc was suspended in distilled water and used for the

characterization of size and zeta potential. The lyophilized pellet was used for other characterization studies.

2.4. Animals

For pharmacokinetics and bioavailability studies male Sprague Dawley rats (n=18) were purchased form Veterinary Research Institute, Lahore and shifted to the animal's house facility at Department of Pharmacy Quaid-i-Azam University Islamabad. The rats were acclimatized for one week in the animal's house. For toxicity evaluation albino mice (n=54) were purchased from National Institute of Health Islamabad (NIH) and shifted to the animal house facility of Department of Pharmacy, Quaid-i-Azam University Islamabad. All the mice were acclimatized for one week in the animal's house. Both studies were performed under the approval of faculty of biological sciences bioethical committee No #BEC-FBS-QAU2018-126. Water and basal diet were provided to the animals ad libitum. Temperature of the animal's house was maintained at 23±3°C. The relative humidity is about to 55±15%. Fresh air circulation is maintained for 12 hours for light and dark cycle with the control light intensity.

2.5. Study Design

2.5.1. Study design for bioavailability/pharmacokinetics study

The study design for bioavailability/pharmacokinetics study was shown in Table 2.1. Animals in the group I and II were given 0.5 ml normal saline solution and aqueous zinc sulfate solution respectively through gavage tube after taking the blood sample at zero time. Whereas animals in the group III were given 0.5 ml homogenized suspension of zinc sulfate nanoparticles before the withdrawal of blood sample at zero time.

Table 2.1. Study design for the bioavailability/pharmacokinetics study

Group (n=9)	**Drug/treatment schedule**
Group I	0.5 ml NS
Group II	4 mg/Kg zinc sulfate (0.5 ml)
Group III	4 mg/Kg zinc sulfate nanoparticle (0.5 ml)

NS: Normal saline

2.5.2. Study design for safety/toxicity evaluation

The study design for safety and toxicity evaluation was shown in Table 2.2. The albino mice (n=54) were segregated into six groups and administered either normal

saline (0.5 ml) or plain zinc sulphate (40 mg/Kg) or zinc oxide nanoparticles (40 mg/Kg) or zinc sulphate nanoparticles (2mg/Kg) or zinc sulphate nanoparticles (4mg/Kg) or zinc sulphate nanoparticles (40mg/Kg) for three weeks daily.

Table 2.2. Study design for the safety/toxicity evaluation of zinc sulfate nanoparticles

Groups (n=9)	Drug treatment schedule
Normal Saline	0.5 ml NS was administered orally daily for three weeks
Plain $ZnSO_4$ 40mg/Kg	40 mg/kg zinc sulfate solution was administered orally daily for three weeks (0.5 ml)
ZnO nanoparticles 40mg/Kg	40 mg/kg Zinc oxide nanoparticles was administered orally daily for three weeks (0.5 ml)
$ZnSO_4$ Nanoparticles 2 mg/Kg	2 mg/kg Zn sulfate nanoparticles was administered orally daily for three weeks (0.5 ml)
$ZnSO_4$ Nanoparticles 4 mg?Kg	4 mg/kg Zn sulfate nanoparticles was administered orally daily for three weeks (0.5 ml)
$ZnSO_4$ Nanoparticles 40 mg/Kg	40 mg/kg Zn sulfate nanoparticles was administered orally daily for three weeks (0.5 ml)

ZnO: zinc oxide; $ZnSO_4$: Zinc sulphate

2.5.3. Sampling

Blood samples (0.5 ml) were collected from the tail vein of the three rats in each group at 0,0.5,1,2,4,6,8,12,24,36,72, and 120 hours in heparinized tubes for bioavailability/pharmacokinetics study. For toxicity and safety study 0.5 to 1 ml of blood was taken by cardiac puncture from these animals in each group weekly for three weeks and divided into two parts: in EDTA tube for hematological examination; in plain vacutainer for serum chemistry liver, kidney and lungs were isolated from decapitated animals and kept in 10% formalin container for histopathological examination.

2.5.4. Analysis of samples

2.5.4.1. Determination of Plasma zinc concentrations

Standard solutions (0.5, 1.5, 2.0 and 2.5 µg/ml) were prepared by dissolving known of amount of zinc in distilled and deionized water. Standard curve was plotted and used for determination of zinc in plasma (Figure 2.1)

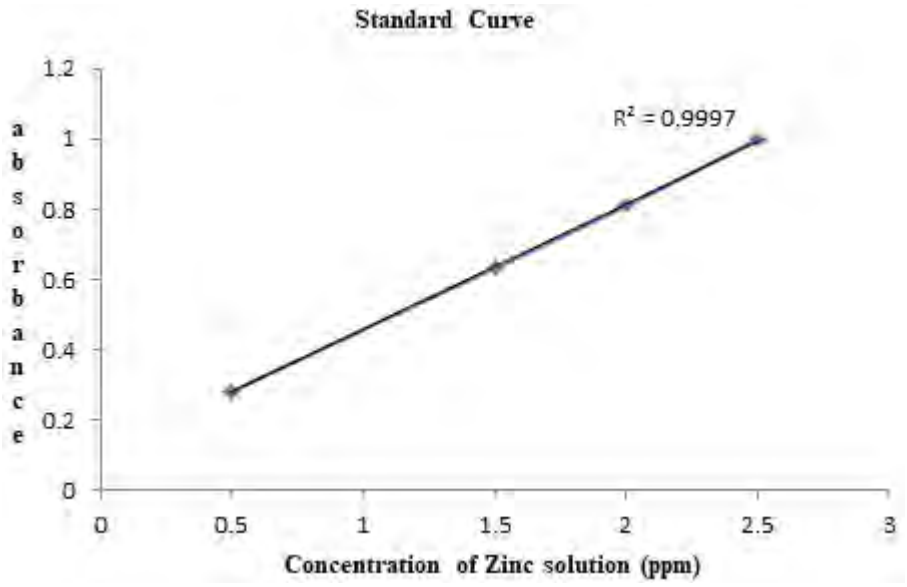

Figure 2.1. Standard curve of zinc sulfate

Plasma sample (100 µl) was diluted to five milliliters with deionized water to be analyzed by Atomic Absorption Spectroscopy using atomic absorption spectrophotometer 55AA from Agilent technologies US. The concentrations obtained in ppm were converted to microgram per milliliter by the following formula:

- Zinc plasma concentration (µg/ml) = Zinc concentration in ppm × 5/0.1

2.5.4.2. Pharmacokinetic parameters

Plasma zinc concentrations (µg/ml) at different time intervals were used to calculate the plasma half-life (hours), maximum plasma concentration C_{max} (µg/ml) , time to reach maximum concentration t_{max} (hours), area under the curve AUC ($_{0\ to120)}$ (µg/ml*h), means residual time MRT (hours) and clearance (ml/hours) by non-compartmental analysis using PK-solver Add ins (Zhang *et al*, 2010).

2.5.4.3. Gross toxicological examination

Animals in different groups were examined for the gross sign of toxicity/pathology and weighed.

2.5.4.4. Hematological examination

Blood samples collected in EDTA tubes were used to determine red blood cell count, white blood cell count, hemoglobin concentration, and differential leukocytes count e.g. neutrophils, eosinophil, lymphocytes and monocytes using Erba Hematology Analyzer, Erba group, USA

2.5.4.5. Serum biochemistry

Serum were separated from the blood by centrifugation and used to determine serum creatinine, bilirubin, total protein, alkaline phosphates (ALP), aspartate transferase as per instruction manual of the manufacturer using Humalyzer 3000® from Human Diagnostics, Germany.

i) Total serum protein

Total serum protein was determined by using specific kit from Human Diagnostics. The kit contained: the reagent composed of: sodium hydroxide 200 mM/L; potassium sodium tartrate 32 mM/L; copper sulphate 12 mM/L; potassium iodide 30 mM/L; standard solution contained protein 80 g/L and sodium azide 0.095%

The standard solution (20 µl) was mixed with 1000 µl of the reagent and incubated for 10 minutes at 25°C. Absorbance of the solution was measured at 546 nm using the Humalyzer 3000®. The same procedure was applied for the serum standard curve and calculation was performed automatically by the instrument with the following formula

- Protein (g/dl) = absorbance of sample / absorbance of standard × 8

ii) Serum bilirubin

Serum bilirubin was measured by the photometric methods using kit from Human Diagnostics containing: DCA (0.3 mM/L of 2, 4-dichloroaniline); 95 mM/L of hydrochloric acid and 70 g/L of detergent. NIT solution contained 2.5 mM of sodium nitrate. BLK solution composed of 1.5 mM/L OF 2,4-dichloroaniline; 57.5 mM/L hydrochloric acid and 35 g/L of detergent. Working reagent was prepared by mixing DCA and NIT in 1:1 ration and allowed to stand for 15 minutes in dark.

The BLK was mixed with the working regent and kept for 10 minutes at 20-25°C in dark. Its absorbance was measured at 546 nm using Humalyzer 3000®. The serum sample (100 µl) was mixed with the working reagent and treated as that of BLK. Absorbance of the sample was measured on same parameter using the same analyzer. Serum bilirubin was measured by the following formula

- Bilirubin (mg/dl) = Δ absorbance of sample × 12.5

  Δ Absorbance of sample = absorbance of sample – absorbance of blank

iii) Alanine aminotransferase (ALT)

Serum ALT level was measured by kinetic method using the kit from the Human diagnostics. It contained: buffer/enzymes reagent (BUF) composed of 125 mM/L of TRIS buffer (pH 7.4); 625 mM/L of l-alanine; LDH (≥ 1.5 kU/L) and 0.095% of of sodium azide. Substrate (SUB) solution composed of: 2-oxoglutarate (75 mM/L); 0.9 mM of NADH and 0.095% of sodium azide (0.095%)

Two ml of the SUB solution was added to 8 ml of the BUF solution and mixed thoroughly to prepare reagent. The serum (200 µl) was added to 1000 µl of the BUF solution and incubated for five minutes at 37°C. then 250 µl of the SUB solution was added. After proper mixing, absorbance was measured at 365 nm exactly after one and two minutes against air (decreasing absorbance) using Humalyzer 3000®. The ALT level was measured as:

- ALT (U/L) = Δ Absorbance / min × 2184

iv) Serum creatinine

Serum creatinine was measured through photometric test by kinetic measurement using kit from Human Diagnostics. The kit contained: 26 mM/L of picric acid solution and 1.6 mM/L of sodium hydroxide. Creatinine (standard) solution contained 176.8 mM/L. sodium hydroxide solution was diluted with the distilled water in ratio of 1+7. Picric acid solution was mixed with diluted sodium hydroxide solution in 1:1 ratio

Standered (100 µl) was mixed with 1000 µl of working reagent at 37°C. absorbance was measured at 490 nm after 30 second (A1) and then exactly after two minutes (A2) using Humalyzer 3000®. Similarly, 100 µl of the sample was added to the 1000 µl of the working reagent and the A1 and A2. of the serum sample were measured at 490 nm with the same parameter. The serum creatinine concentration was determined as

- Serum creatinine (mg/dl) = (A1-A2) of sample / (A2-A1) of blank × 2.0

v) Aspartate Aminotransferase

Aspartate Aminotransferase (AST) was measured by using the kit from human diagnostics. The kit composed of: AST Assay Buffer, 25 mL, AST Enzyme Mix, AST Developer, AST Substrate, Glutamate Standard, 0.1 M ,0.1 mL AST Positive Control. The Master Reaction Mix i.e. AST Assay Buffer 80 µL, AST Enzyme Mix 2 µL, AST Developer 8 µL, AST Substrate 10 µL, the 100 µL of the Reaction Mix was used for reaction.

100 µL of the Reaction Mix was added to each well and then Incubate at 37 °C. After 2–3 minutes, take the initial measurement (Tinitial) with the absorbance at 450 nm at the initial time (A450) initial. Then mixture was incubated at 37 °C while taking measurements (A450) every 5 minutes and then Continue taking measurements until the value of the most active sample is greater than the value of the highest standard. Calculate The final measurement [(A450) final].

- By using the formula $\Delta A450 = (A450)\ final - (A450)\ initial$
- AST Activity = B × Sample Dilution Factor (Reaction Time) × V

  B = Amount (nmole) of glutamate generated between Tinitial and Tfinal,
  Reaction Time = Tfinal – Tinitial (minutes),
  V = sample volume (mL) added to well.

vi) Alkaline phosphatase

Alkaline phosphatase (ALP) was measured by using the kit from Human Diagnostics. Components of kits were as follows Test.-Buffer 1 ml (pH 10 for alkaline, pH 4.9 for acid phosphatase), is added to 1 ml substrate (M / 100 Na 2-phenyl phosphate). The reaction mixture was warmed at 37° C for three minutes, 0.1 ml plasma was added and mixed. The solution is incubated at 37° C, 15 min. for alkaline phosphatase (one hour for acid). Then 0.8 ml N/2 sodium hydroxide was added for alkaline phosphatase (1 ml N/2 sodium hydroxide for acid), and 1.2 ml. M/2 sodium bicarbonate was added for alkaline phosphatase (1 ml. M/2 $NaHCO_3$ for acid). Then 1 ml 0.6% 4-amino-antipyrine was added and mixed. Finally, 1 ml 2.4% ferrocynate was added and mixed. ALP was calculated as

- ALP (K.-A. units 100 ml) = $\frac{\text{Test -Control}}{\text{Standard -Blank}} \times 10$

2.5.4.6. Histopathological examination

The collected tissues were processed for histopathological examination. Liver, kidney and lungs were prefixed in 10% formalin for histopathological examination. The fixed tissues were trimmed, dehydrated, embedded in paraffin, sectioned, mounted on glass slides and then stained with hematoxylin and eosin for histopathological examination under light microscope.

2.6. Statistical analysis

All results were reported as mean ± standard error. Data obtained were analyzed by student'S t-test for bioavailability and pharmacokinetics parameters whereas plasma concentration, hematological and serum biochemistry parameters were analyzed by one-way ANOVA/ and two-way ANOVA using Duncan and LSD test for multiple comparisons respectively. All the statistical analyses were performed using IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp. The p-value < 0.05 was used to assign the level of significance.

CHAPTER 3

RESULTS

3. RESULTS:

3.1. Characterization of the Nanoparticles

Average size of the nanoparticles was determined by zetasizer is shown in Figure 3.1. The average range of the particles size is 240.5 nm.

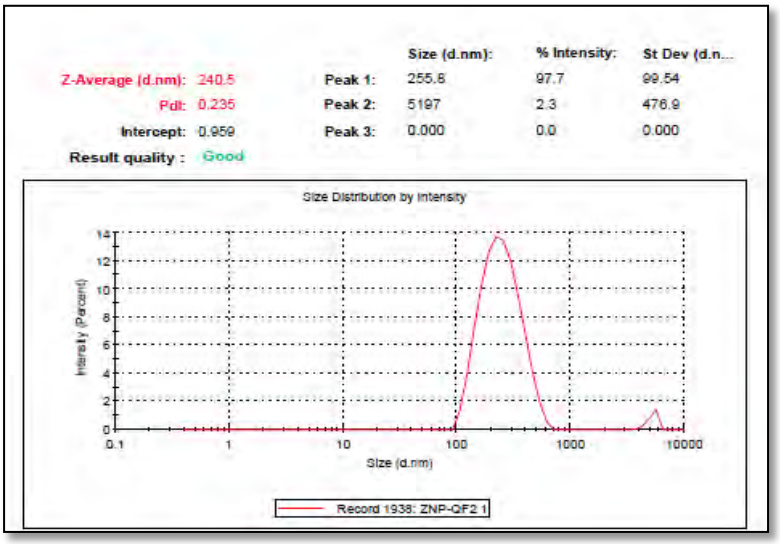

Figure 3.1. Particle size of zinc nanoparticles

Zeta potential of the nanoparticles determined by the zetasizer is shown in figure 3.2. Zeta potential of the particles was found to be 32.8 mV.

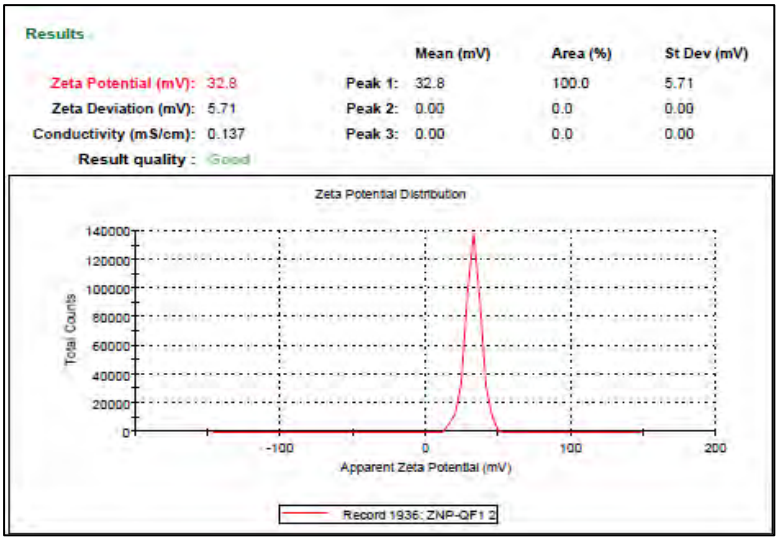

Figure 3.2. Zeta potential of zinc nanoparticles

3.2. Plasma zinc concentration

The plasma concentration in animals of various groups is shown in Table 3.2 and Figure 3. From the administration of drug till one hour, no significant difference was observed in the plasma zinc concentration of animals. However, after one-hour

significant increase was observed in the plasma zinc concentration of animals receiving zinc supplements than the animals given normal saline. Later on animal receiving zinc nanoparticles showed significantly higher zinc plasma concentration than that of animals received plain zinc formulation. The increase remained significant till the end of the study (120 hours).

Table 3.1. Plasma zinc concentrations at different time points

Time (Hours)	ZnP (µg/ml)	ZnN (µg/ml)	N/S (µg/ml)
0	11.41 ±0.37[a]	11.62 ±0.62[a]	11.10 ± 0.31[a]
0.5	13.77 ± 0.82[a]	13.23 ±0.68[a]	11.53 ± 0.15[a]
1	12.47 ± .15[b]	13.55 ±.46[a]	11.37 ± .29
2	11.60 ±.28[b]	19.06 ±.1.5[a]	13.5 ±.74[b]
4	14.14 ±.21[b]	16.41±.33[a]	12.17 ±.60[c]
6	11.52 ±.24[b]	18.84 ±.74[a]	12.57 ±.58[b]
8	11.30 ±0.90[b]	18.13 ±.41[a]	10.57 ±.52[b]
12	10.71 ±.1[b]	15.68 ±1.28[a]	11.10 ±.70[b]
24	12.54 ±.81[b]	16.11 ±1.08[a]	12.33 ±.52[b]
36	12.93 ±.34[b]	15.73 ±.55[a]	12.30 ±.15[b]
72	11.40 ±.35[b]	15.27 ±.57[a]	11.33 ±.17[b]
120	10.73 ±.53[b]	13.61 ±.55[a]	9.83 ±.33[b]

Each value represents mean±SE (n=3) of plasma zinc concentration. ZnP: Plain zinc formulation, ZnN: Zinc nanoparticles. Values having different supercript in same rows are significantly different ($p<0.05$)

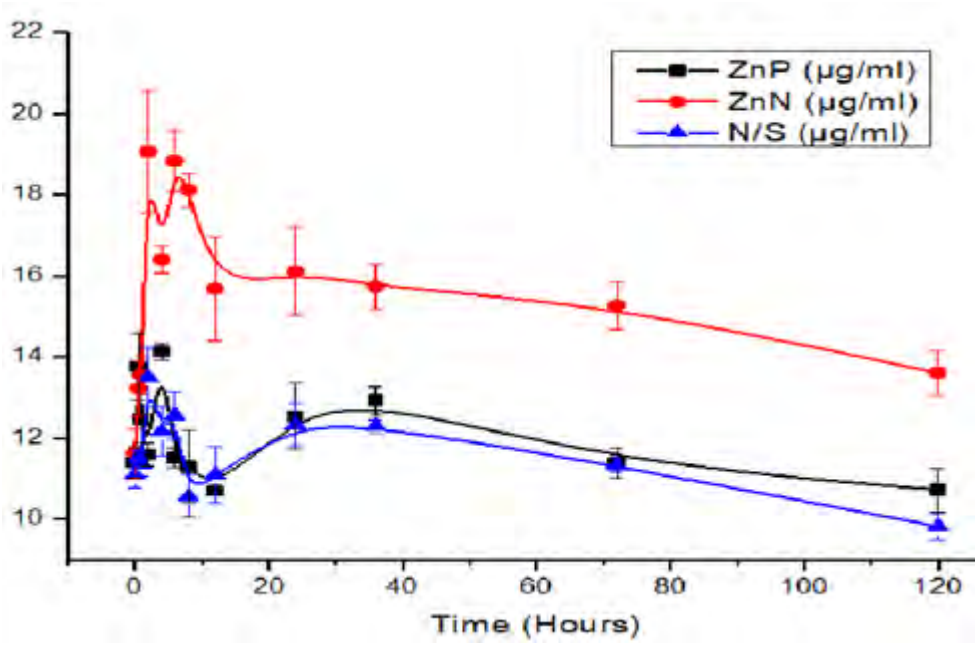

Figure 3.3. Zinc plasma concentration vs time curve

3.3. Pharmacokinetics Parameters

Area under the curve 0-120 hour in the animals receiving zinc formulation is shown in the Table 3.1. Area under the curve in the animals is significantly higher than the animals given plain zinc formulation. Similarly significant rise was observed in plasma half-life, C_{max} , and MRT in the animals given zinc nanoparticles than that of animals received plain zinc formulation. Furthermore, significant decrease was exhibited in t_{max} and clearance in animals received zinc sulfate nanoparticles than that of amount given plain zinc sulfate solution.

Table 3.2. Pharmacokinetics parameters in mice received various formulations

Parameters	**ZnP**	**Zn N**	**p-value**
Half-life (h)	236.63± 12.30	540.13± 20.91	0.000
t_{max} (h)	3.50± 0.29	2.17 ± 0.17	0.026
C_{max} (µg/ml)	14.68 ± 0.37	19.50 ± 1.09	0.03
AUC $_{0-120}$ (µg/ml*h)	1404.4 ± 27.84	1837.36 ± 17.49	0.000
MRT (h)	473.63 ± 28.69	681.05 ± 25.81	0.043
Cl (ml / h)	0.11 ± 0.01	0.03 ± 0.01	0.001

Each value represent mean ± S.E (n=3) of half-life, C_{max} (Peak plasma concentration) .AUC $_{0-120}$, (Area under curve), MRT (Mean residual time), t_{max} (time to reach C_{max}) and Cl (Clearance) were analyzed by Student's t-test (p >0.05). ZnP: plain zinc sulphate and ZnN : Zn sulphate nanoparticles.

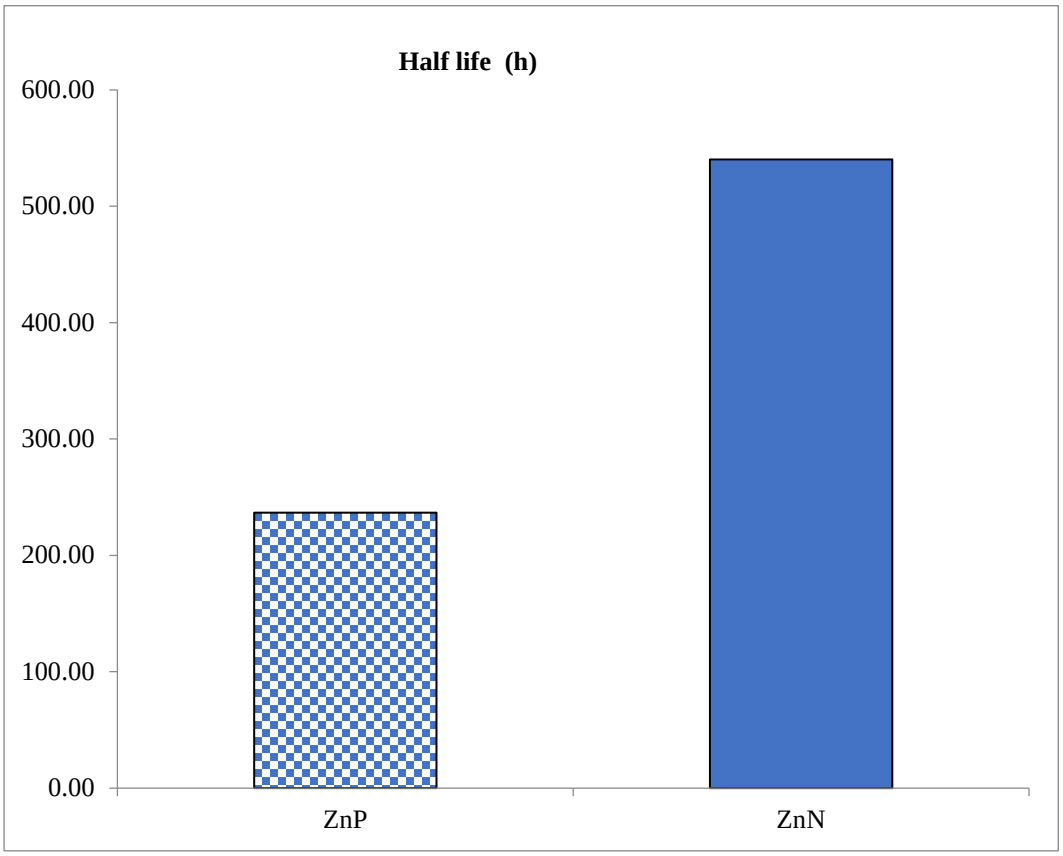

Figure 3.4. Half-life of Plain zinc sulphate and zinc sulfate nanoparticles

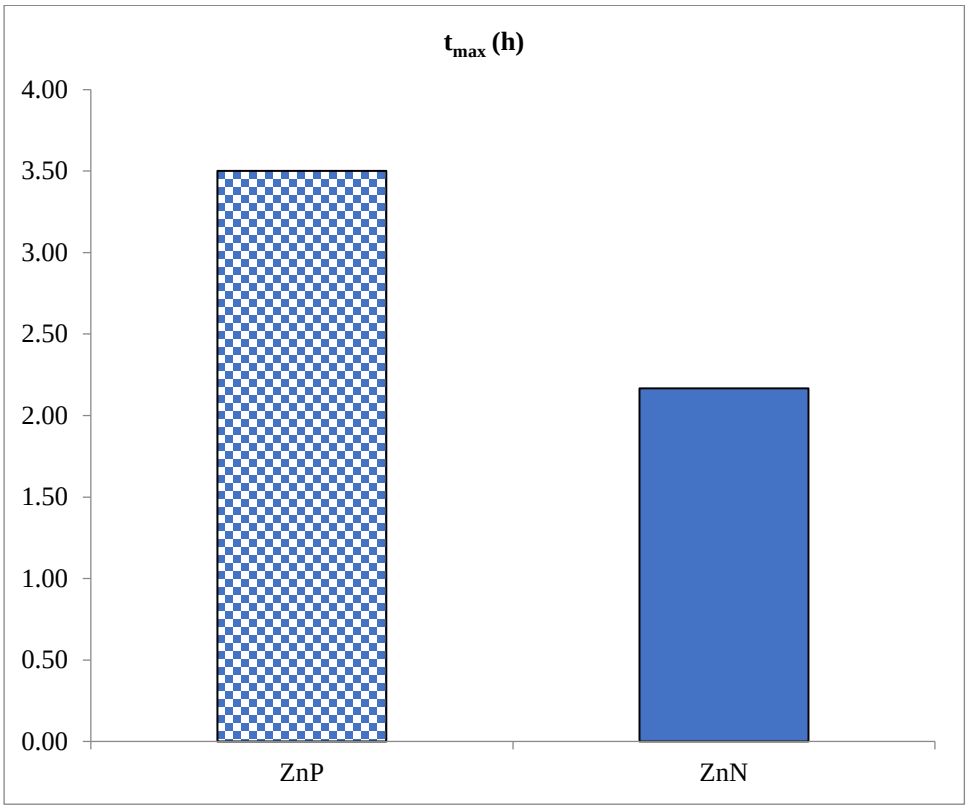

Figure 3.5. $t_{max:}$ (time to reach peak plasma concentration) of Plain zinc sulphate and zinc sulfate nanoparticles

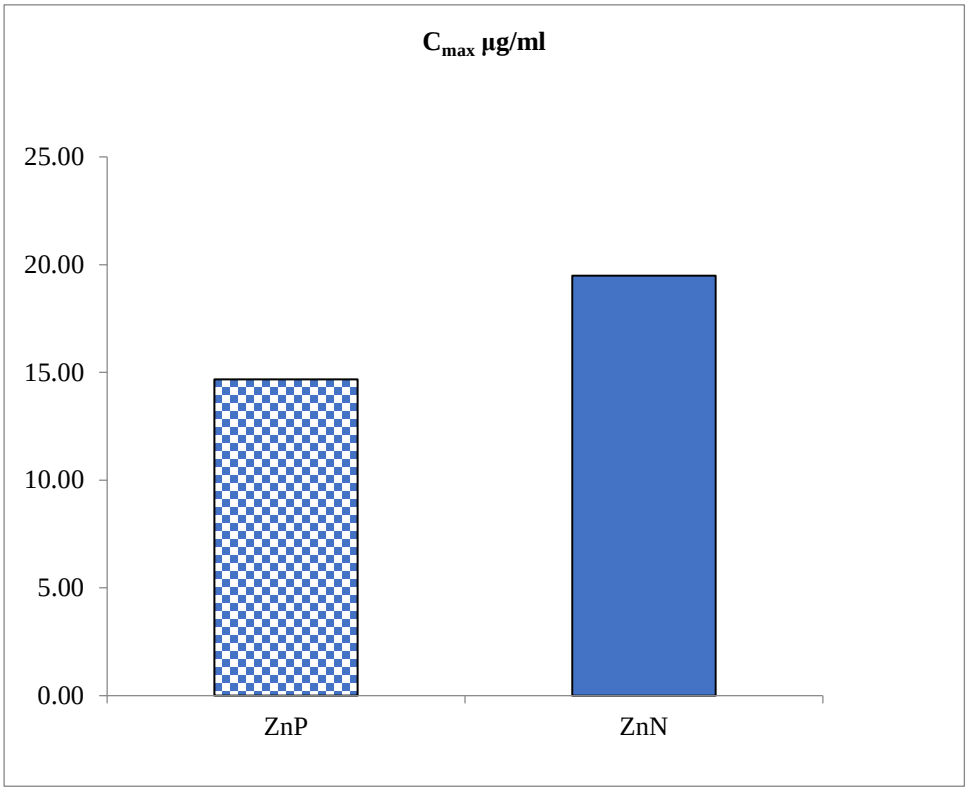

Figure 3.6. C_{max} (peak plasma concentration) of Plain zinc sulphate and zinc sulfate nanoparticles

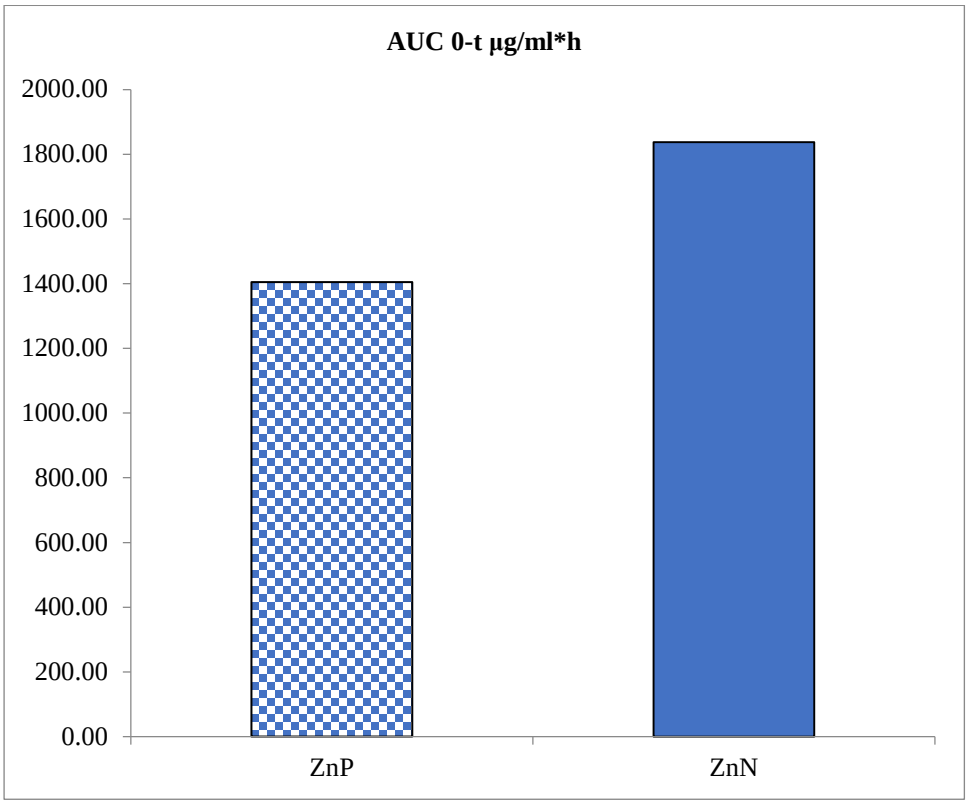

Figure 3.7. AUC (Area under curve) of Plain zinc sulphate and zinc sulfate nanoparticles

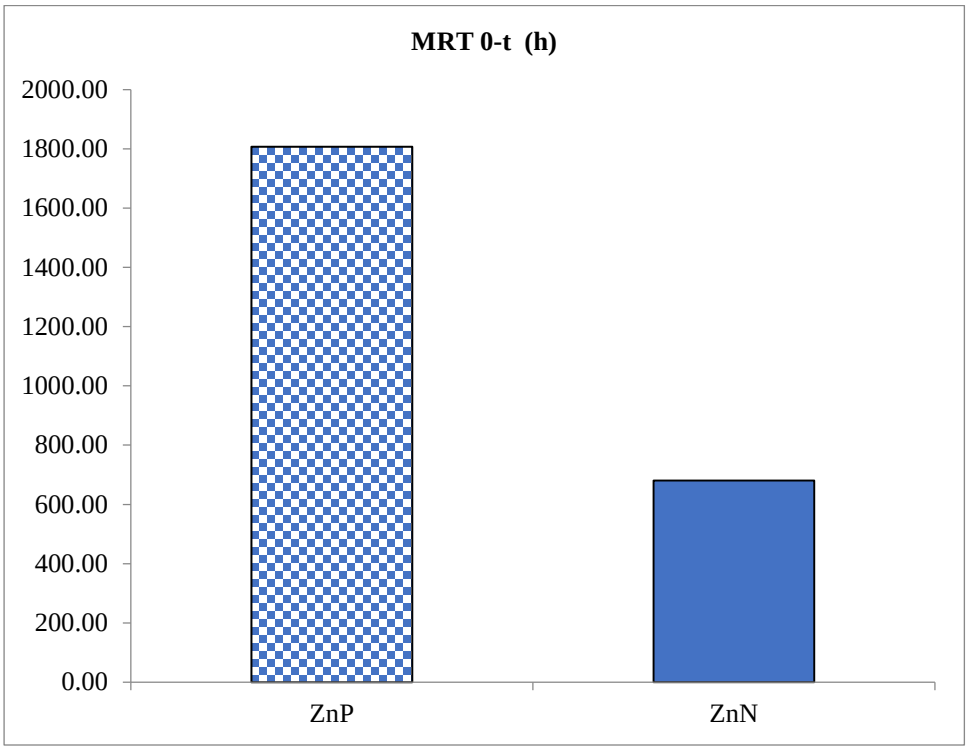

Figure 3.8. MRT (Mean residual time) of Plain zinc sulphate and zinc sulfate nanoparticles

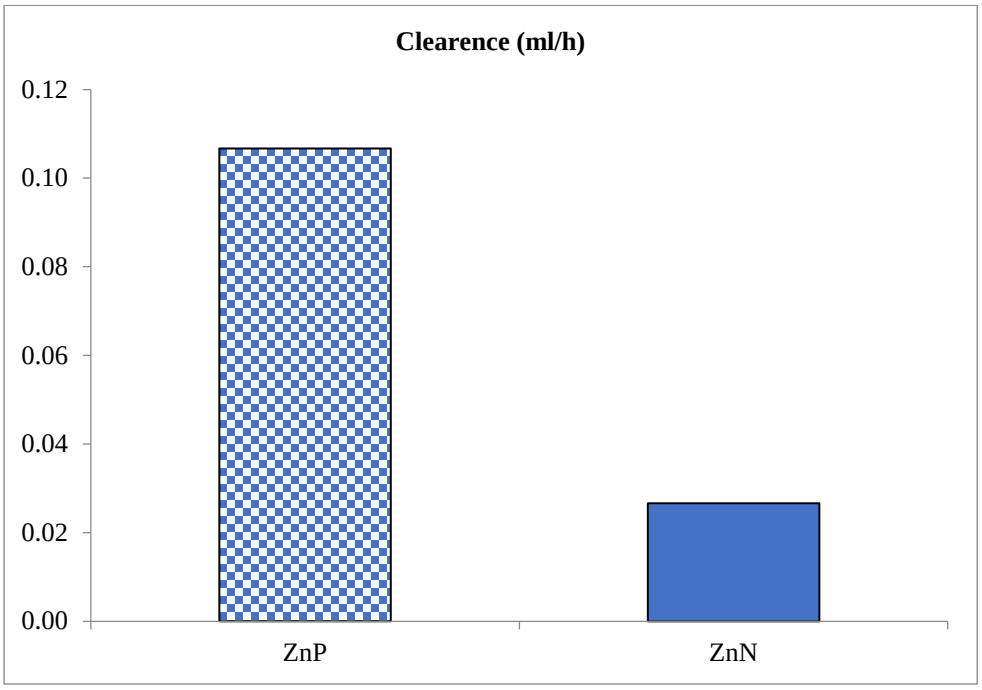

Figure 3.9. Clearance of Plain zinc sulphate and zinc sulfate nanoparticles

3.4. Toxicological Evaluation

Toxicological evaluation was carried out for zinc sulfate nanoparticles. For this purpose, hematological, biochemical and histopathological assessment were performed which are as follow:

3.4.1. Hematological examinations

Hematological parameters are shown in Table 3.3. Hematological values were not affected very significantly. However significant decrease in hemoglobin, RBC count was observed in animals given zinc oxide nanoparticles.

Table 3.3 Effect of various treatments on the hematological parameters at 21st day of Study

Group	Hb	WBCs	RBC	Neutrophils	Lymphocytes	Eosinophils	Monocytes
NS 0.5ml	12.7±0.98[a]	2830±132.7[a]	8.5±0.57[a]	53.00±9.81[a]	43±4.04[a]	2±0.2[a]	2±0.57[a]
ZnP 40mg	13.7±0.30[a]	8230±1079[b]	9.26±0.54[a]	60.00±5.77[a]	36±2.3[a]	1.6±0.2[a]	2±1.0[a]
ZnO 40mg	8.8±0.57[b]	5530±98.14[b]	5.60±0.34[b]	28.00±4.61[b]	70±11.54[b]	1±0.28[b]	1±0.3[b]
ZnN 2 mg	12.9±1.09[a]	9190±519.7[b]	9.1±0.63[a]	12.00±2.88[b]	86±8.08[b]	1±0.17[a]	1±0.20[a]
ZnN 4 mg	8.7±0.86[b]	2410±109.6[a]	4.5±0.51[b]	20.00±10.46[b]	78±16.16[b]	1±0.01[a]	1±0.11[a]
ZnN 40 mg	7.8±0.43[b]	2400±115.4[a]	4.4±173.2[b]	17.00±4.8[b]	80±2.8[b]	1.2±0.3[a]	1±0.40[a]

Each value represents mean±SE (n=3) of RBC: Red Blood Cells, WBC: White Blood Cells, HB: Hemoglobin, NS: Normal saline, ZnP: Plain zinc sulphate, ZnO: zinc oxide nanoparticles. ZnN: Zinc sulphate nanoparticles. Values having different superscripts in columns are significantly different ($p<0.05$).

3.4.2. Biochemical analysis

Table 3.4 depicts the serum total protein and creatinine concentration in mice received various treatments on 7th, 14th and 21st day of the study

Table 3.4. Effect of various treatments on serum protein and creatinine in mice

	Serum total protein			Serum creatinine		
Treatments	7 days	14 days	21 days	7 days	14 days	21 days
NS 0.5ml	4.03±1.1[a]	4.03±1.1[a]	4.03±1.1$_a$	.50±0.30[a]	.50±0.3[a]	.50±0.30[c]
ZnP 40mg	3.70±0.5[a]	2.10±0.1[b]	2.63±0.37[a]	.40±0.10[a]	.63±0.41[a]	1.40±0.43[b]
ZnO 40mg	2.06±0.15[b]	2.03±0.6[c]	1.30±0.45[b]	.83±0.35[a]	3.40±0.90[b]	4.86±0.95[a]
ZnN 2 mg	3.70±0.7[a]	3.66±1.5[a]	3.06±0.60$_a$	.40±0.10[a]	1.16±0.32[a]	1.46±0.57[b]
ZnN 4 mg	4.06±0.6[a]	4.23±0.2[a]	5.50±0.6[a]	1.43±0.05[a]	1.52±0.21[a]	1.47±0.41[b]
ZnN 40 mg	5.40±1.2[a]	5.10±0.9[a]	5.20±1.9[a]	1.60±0.10[a]	1.66±0.57[a]	1.83±0.1[b]

Each value represents mean ± S.E (n=3). NS: Normal saline, ZnP: Plain zinc sulphate, ZnO: zinc oxide nanoparticles. ZnN: Zinc sulphate nanoparticles. Values having different superscripts in columns are significantly different ($p<0.05$).Values having different superscripts in columns are significantly different ($p<0.05$).

Serum bilirubin and alanine transferase level in mice receiving carious treatments at day 7, 14 and 21 of the study are depicted in Table 3.5.

Table 3.5. Effect of various treatments on serum bilirubin and alanine transferase (ALT) in mice

	Serum bilirubin			Serum alanine transferase (ALT)		
Treatments	7 days	14 days	21 days	7 days	14 days	21 days
NS 0.5ml	1.06±0.37[b]	.91±0.08[b]	.62±0.45[b]	43±23.0$_b$	32±11.5[b]	32.33±11.5[b]
ZnP 40mg	.81±0.20[b]	.71±0.10[b]	.56±0.90[b]	27.6.2[b]	42.3±21.9[b]	29.33±14.0[b]
ZnO 40mg	7.98±2.71[a]	8.60±1.6[a]	7.10±1.0[a]	372±86.0[a]	500±170.0[a]	578±214[a]
ZnN 2 mg	1.45±0.43[b]	1.26±0.5[b]	1.11±0.18[b]	35.33±10.5[b]	25±10.58[b]	31.67±5.8[b]
ZnN 4 mg	1.56±0.41[b]	.87±0.1[b]	.90±0.90[b]	43.33±7.6[b]	39.66±5.0[b]	48±8.0[b]
ZnN 40 mg	1.27±0.54[b]	1.12±0.3[b]	1.61±0.17[b]	29.66±9.0[b]	35±14.7[b]	31±21[b]

Each value represents mean ± S.E (n=3). NS: Normal saline, ZnP: Plain zinc sulphate, ZnO: zinc oxide nanoparticles. ZnN: Zinc sulphate nanoparticles. Values having different superscripts in columns are significantly different ($p<0.05$).Values having different superscripts in columns are significantly different ($p<0.05$).

Table 3.6 describes serum concentration of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in mice given various treatments at 7th, 14th, and 21st day of the study.

Table 3.6.. Effect of various treatments on serum AST and ALP in mice

	Serum aspartate aminotransferase (AST)			Serum alkaline phosphatase (ALP)		
Treatments	7 days	14 days	21 days	7 days	14 days	21 days
NS 0.5ml	33.3±16.50^{c}	33.3±16.5^{c}	33.3±16.5^{c}	31±11^{a}	31±11^{b}	31±11^{c}
ZnP 40mg	25.33±8.5^{c}	38.66±6.4^{c}	32.66±6.3^{c}	18.33±4.5^{a}	17±6^{b}	56±11.5^{b}
ZnO 40mg	312±42^{a}	238±32^{a}	204±4.6^{a}	45±6.3^{a}	97±12^{a}	204±4^{a}
ZnN 2 mg	21±8.5^{c}	49±23^{c}	43.6±10.5^{c}	45.3±1.5^{a}	30±15^{b}	60 ±12^{b}
ZnN 4 mg	35.6±7.57^{c}	45.66±11^{c}	54±14^{c}	45±0.4^{a}	27±7.5^{b}	43.3±9.45^{b}
ZnN 40 mg	64±14^{b}	57±15^{b}	50±20^{b}	45±0.35^{a}	39±5.5^{b}	46.66±15.2^{b}

Each value represents mean ± S.E (n=3). NS: Normal saline, ZnP: Plain zinc sulphate, ZnO: zinc oxide nanoparticles. ZnN: Zinc sulphate nanoparticles. Values having different superscripts in columns are significantly different ($p<0.05$).Values having different superscripts in columns are significantly different ($p<0.05$).

Animals receiving zinc oxide nanoparticles indicated significant rise in serum bilirubin, ALT, AST, ALP and creatinine than that of animals given normal saline. Whereas significant decline was observed in total serum protein in these animals as compared to animal receiving normal saline. However, animals given zinc nanoparticles indicate significantly improved serum biochemistry as compared to animals given zinc oxide nanoparticles. But, animals receiving higher dose of zinc sulfate nanoparticles (40 mg) showed increase in serum creatinine, AST, ALT, and ALP than that of animals given normal saline. Although the rise in these parameters were not comparable to the animals given zinc oxide nanoparticles.

3.4.3. Histopathological evaluation

3.4.3.1. Effect of different treatments on kidney

The effects of various treatment on kidney at 7, 15, 21 days of the treatment are show in Figure 3.10, 3.11 and 3.12. Figure 3.10 A depicted the kidney histology of the animals given normal saline. Kidney parenchyma with normal glomeruli was evident in the figure indicating no histopathological damage at day 7. Whereas animal given

zinc oxide nanoparticles indicated mild disruption in the glomerulus structure. With continued treatment, sever degenerative changes were observed in the kidney parenchyma with fewer number of glomeruli (Figure 3.10 F, 3.11 F and 3.12 F). Animals given zinc sulfate nanoparticles indicated normal parenchyma of kidney with more number of glomerulus and normal columnar epicedial cells at day 7, 15 and 21 (Figure 3.10 E, 3.11 E and 3.12 E).

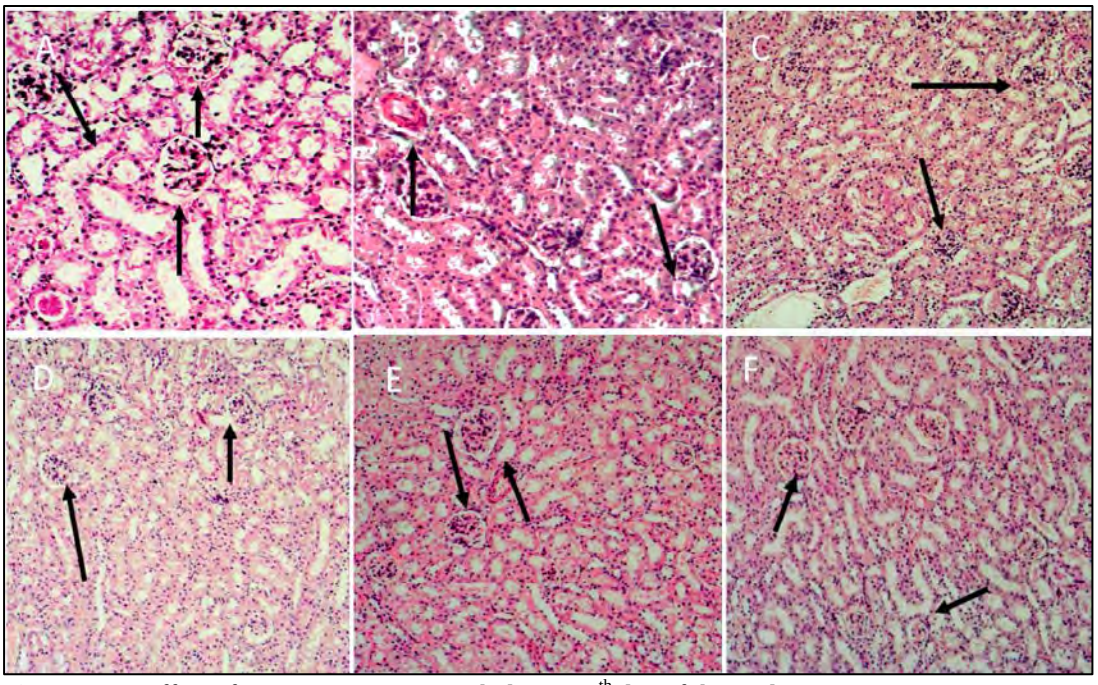

Figure 3.10. Effect of various treatments on kidney at 7th day of the study

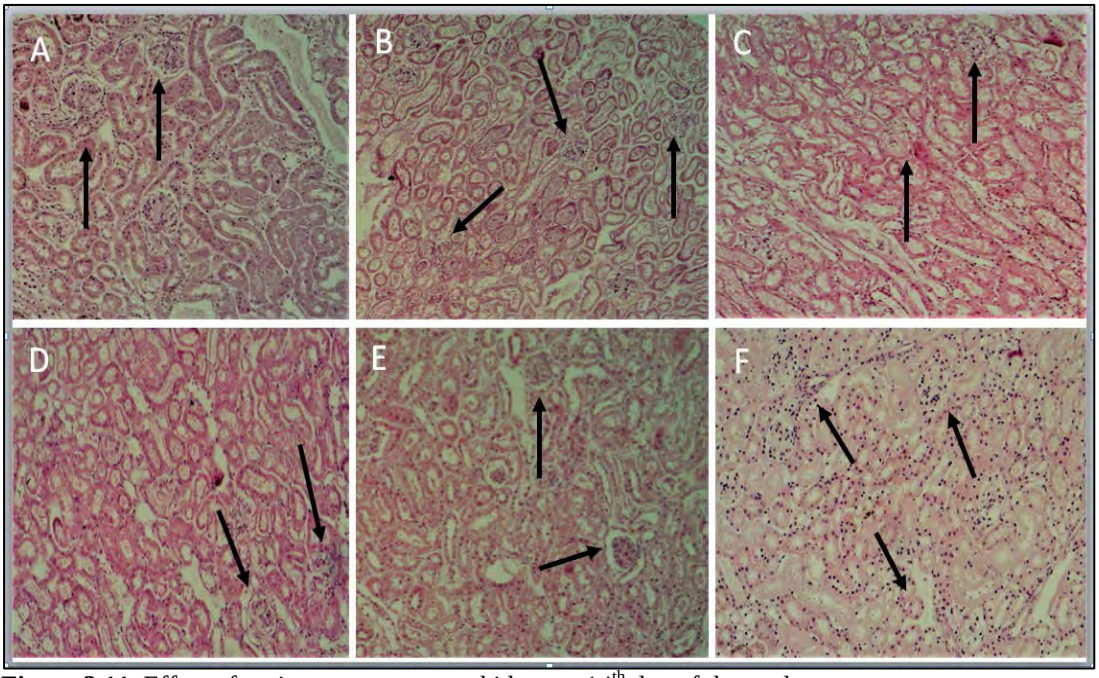

Figure 3.11. Effect of various treatments on kidney at 14th day of the study

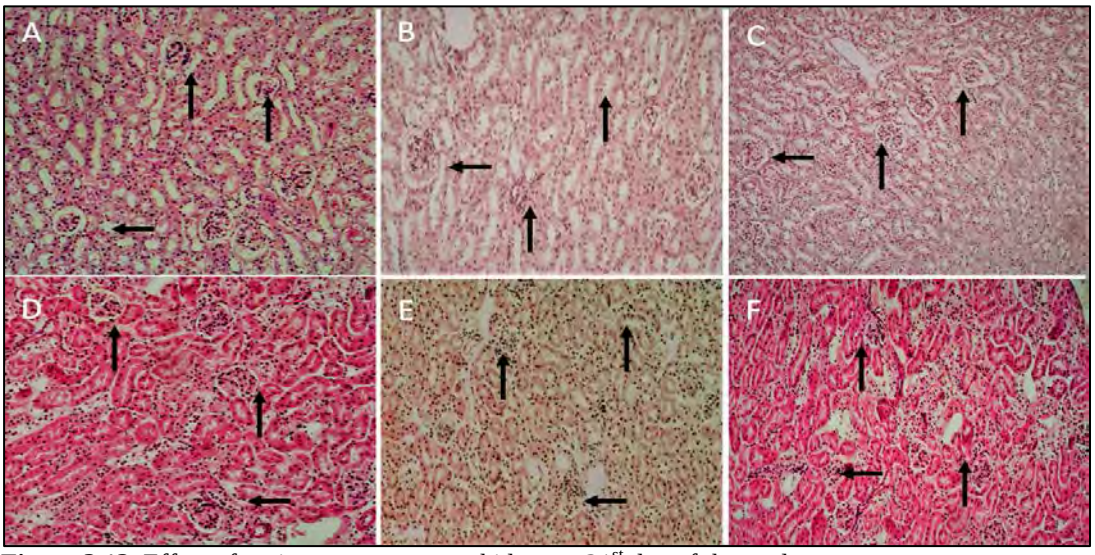

Figure 3.12. Effect of various treatments on kidney at 21st day of the study

3.4.3.2. Effect of different treatment on liver

Effect of various treatments on liver histology is shown in figure 3.13, 3.14 and 3.14 Administration of zinc oxide in animals cause mild degenerative changes in the liver architecture after 7 days of treatment. Later on, sever inflammatory changes with infiltration of inflammatory cells were observed. Onward 21 days disruption of sinusoids and necrosis of hepatic cells were observed as shown in figure 3.13 F, 3.14 F and 3.15 F. Whereas animals given low doses of zinc sulfate nanoparticles did not indicate such degenerative changes (Figure 3.13 D, 3.14 D, 3.15 D). However, animal given higher dose of Zn nanoparticles showed mild deteriorating sign with disrupted hepatocytes but normal rosette arrangement of the cells (Figure 3.13 E, 3.14 E and 3.15.E)

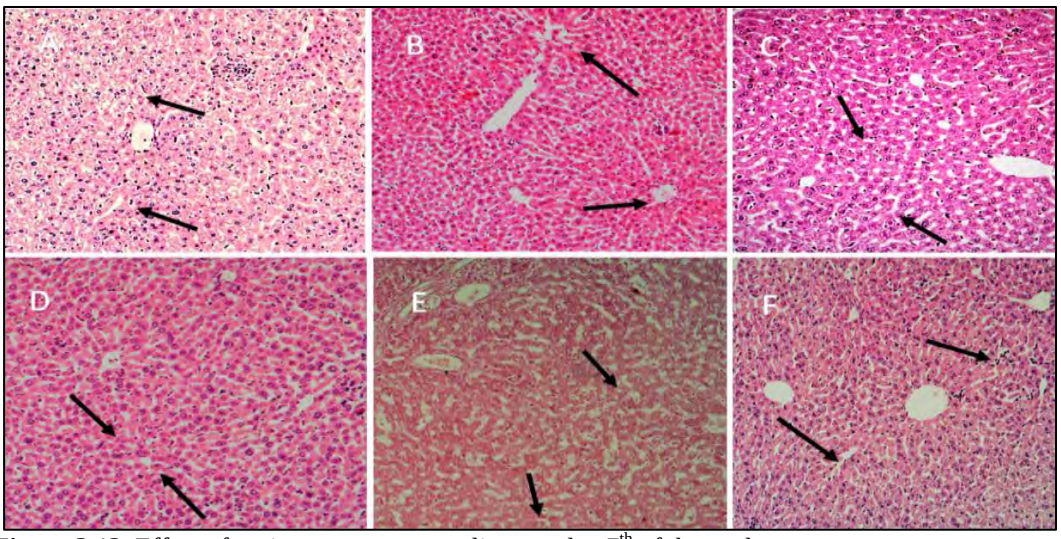

Figure 3.13. Effect of various treatments on liver on day 7th of the study

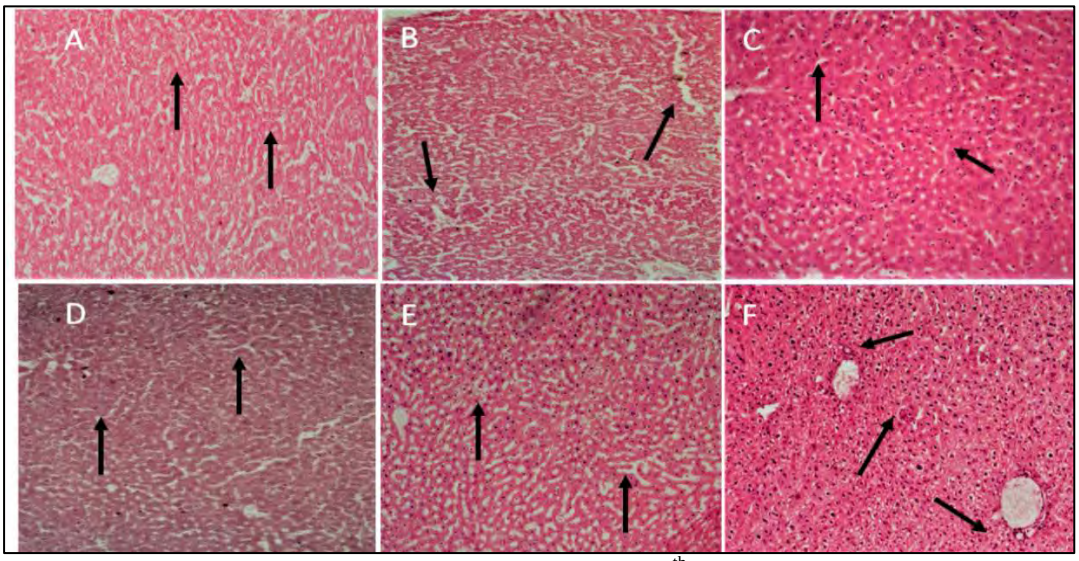

Figure 3.14. Effect of various treatments on the liver at day 15th of the study

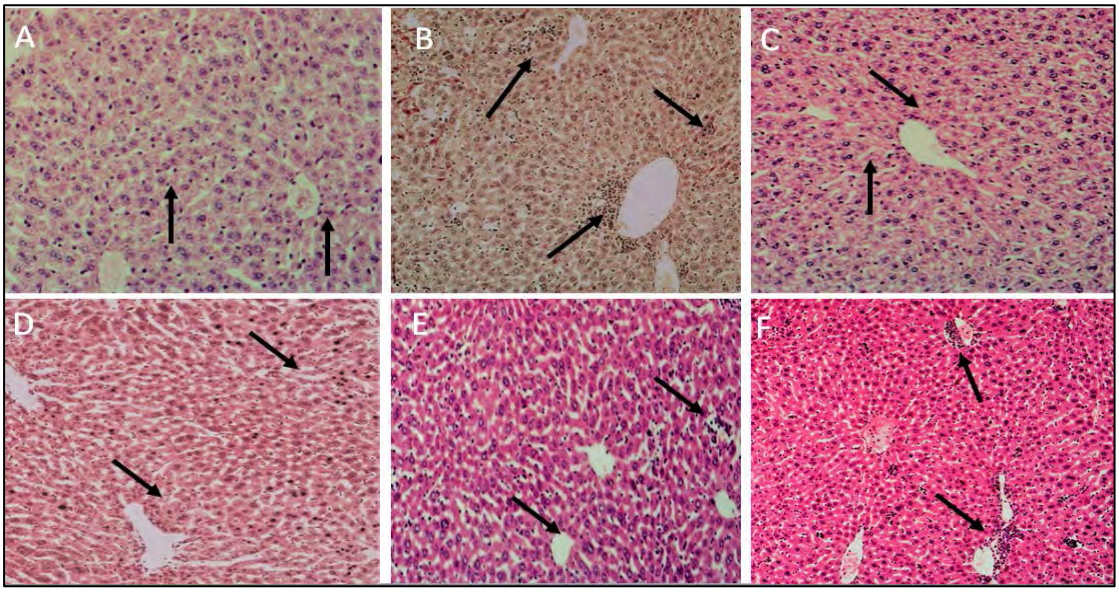

Figure 3.15. Effect of various treatment on liver at day 21st of the study

3.4.3.3. Effect of different treatments on lungs

Effect of various treatments on lungs histology is shown in figure 3.16, 3.17 and 3.18. Animals receiving zinc oxide nanoparticles indicated progressive damage on lung histology with lesser number of alveoli and damage lungs parenchymal cells (Figure 3.16 F, 3.17 F, 3.18 F). However, animals receiving zinc sulfate nanoparticles indicated more number of alveoli and almost normal lungs parenchyma as shown in Figure 3.16 D, 3.17 D and 3.18 D. Animal given higher dose of nanoparticles indicated mild damage to lung tissue and alveoli (Figure 3.16 E, 3.17 E and 3.18 E).

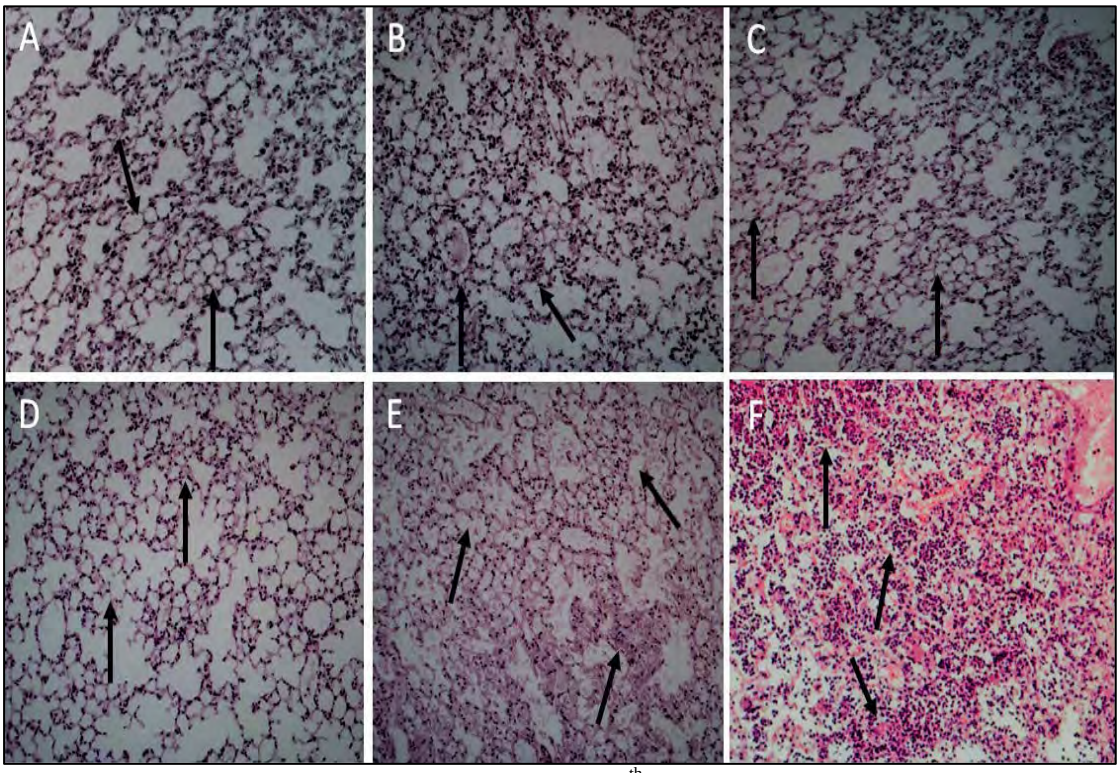

Figure 3.16. Effect of various treatments on lungs at day 7th of the study

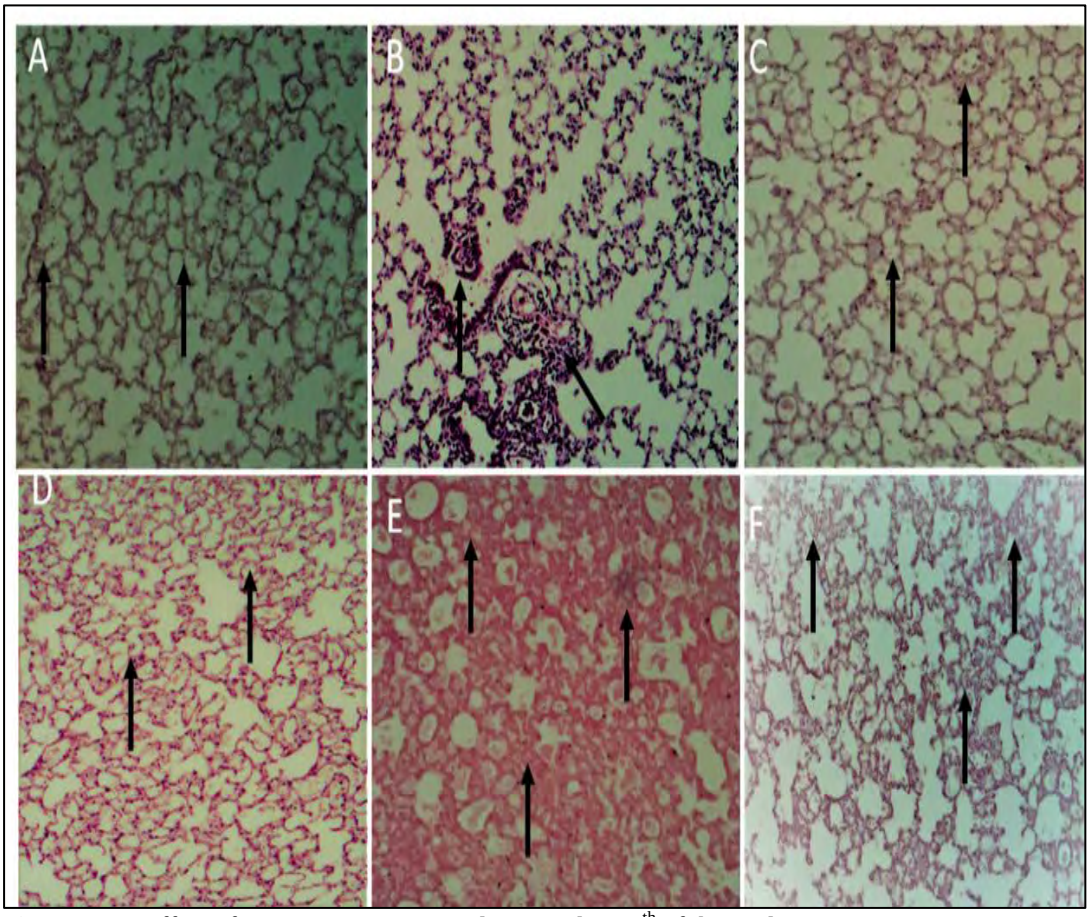

Figure 3.17. Effect of various treatments on lungs at day 15th of the study

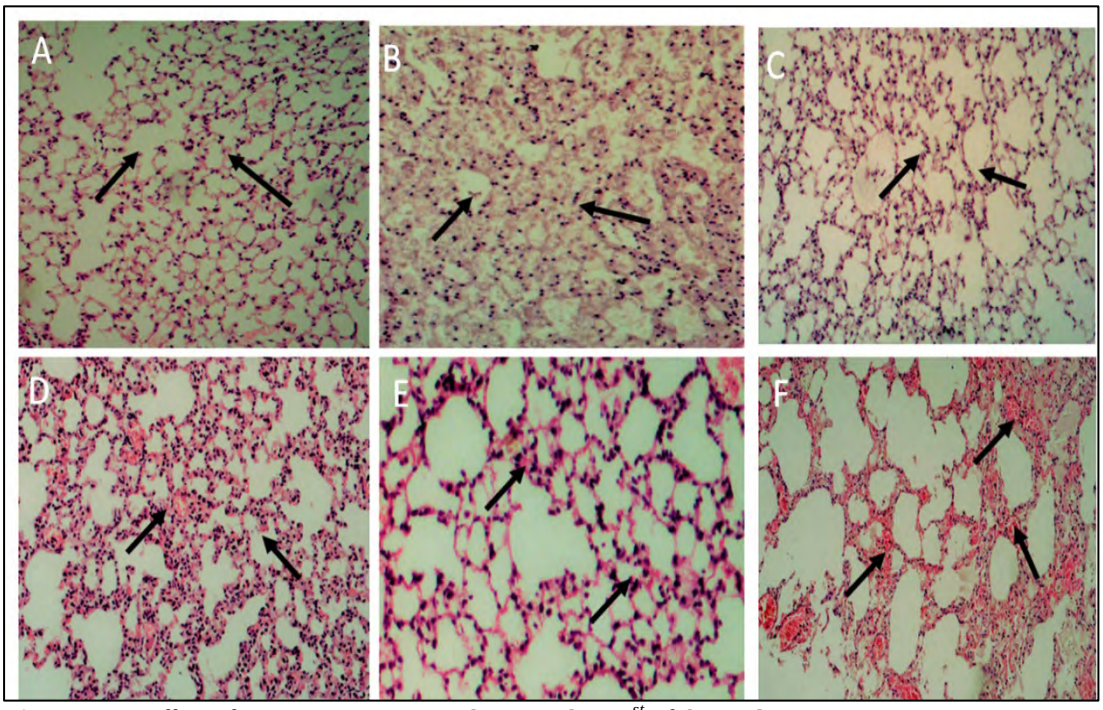

Figure 3.18. *Effect of various treatment on lungs at day 21st of the study*

CHAPTER 4

DISCUSSION

4. DISCUSSION

Bioavailability of zinc is affected due to many factors which includes divalent cations like calcium (Wood, 2000), copper, iron (Solomons and Jacob, 1981), magnesium etc. Phytate rich diet is the another most important factor which contribute to decrease the dietary zinc concentration (Cichy *et al*, 2005). ZIP transporter are mainly involved in the cellular uptake of zinc either from diet or extracellular compartment (Kambe *et al*, 2015b). These receptors get saturated and then zinc absorption occur through para-cellular route (slow). To address the problem, chitosan based zinc nanoparticles were formulated to control the release of zinc from formulation and enhancing its para-cellular movement (Sadzuka et al, 1998). Significant rise in plasma centration of the animals receiving zinc formulation indicate lag time in the zinc absorption to systemic circulation (Zhang *et al*, 2014). Moreover consistent significant increase in the zinc plasma concentration in animals receiving nanoparticles indicate more contact with the absorptive surface hence greater cellular uptake of zinc. Both of these effects are reported in studies involving chitosan as a polymer. Greater plasma concentration can also be explained by lesser saturation of ZIP transporter due to control release phenomenon (Aspden *et al*., 1996).

Maximum concentration after two hour (Table 3.1) indicate faster absorption as compared to reported time in various studies conducted with zinc oxide nanoparticles (Li *et al*, 2012a; Zhang *et al*, 2018a). Moreover plasma zinc concentration in animals given plain zinc formulation show fluctuation at 2, 8 and 12 hours are in line with the reported studies (Hahn and Baker, 1993).

Half-life of animals receiving plain zinc sulfate formulation is 236 hours with area under the curve of 1440.4±27.84 are in accord with previously reported studies. Significant rise in half life and area under the curve of the animals receiving zinc sulfate nanoparticles can be explain by the properties conferred by the polymer (chitosan) and nano nature of zinc particle (Vila *et al*, 2002b). Significantly evaluated in zinc concentration even after 24, 36 and 120 hours indicate presence of zinc at the absorptive site along with the diurnal variation indicate continued absorption of zinc even after single dose. The same is also reflected in significant decrease in clearance in the animals (Hahn and Baker, 1993). These findings are align with other publish studies.

The increased bioavailability of nanoparticles can be explained by control release of zinc when it is formulated as chitosan based nano formulation (Vila et al., 2002b).

Zinc oxide nanoparticles produce toxicity due to the presences of reactive oxygen species. The reactive oxygen species produce oxidative stress which further causes DNA damage as described in other study (Choi *et al.*, 2015b). Animals given zinc oxide nanoparticles showed decrease in RBC count and hemoglobin count (Wang *et al.*, 2008). The same has been reported in the studies conducted by other researches (Kong *et al.*, 2019). Mild leukopenia seen in zinc oxide nanoparticles treated animals may be due to the oxidative stress which is reported in many studies (Esmaeillou *et al.*, 2013). Similarly raised bilirubin, AST, ALT, ALP and creatinine can be explained due to the hepatic cell damage because of oxidizing radicle produce by the zinc oxide nanoparticles. These finding are also supported by the finding of other researchers (Choi *et al*, 2015a; Li *et al*, 2012a; Xia *et al*, 2008).

The negative impact on hematology and serum biochemistry is also reflected in histopathological findings. Damage hepatocytes and disrupted agreement of hepatic cells indicate liver damage by oxidizing species. Moreover damaged columnar epithelium in nephron tubules and decrease in number of glomeruli also supports the increase in the creatinine level. These histopathological findings are also reported by the finding of other scientists (Esmaeillou *et al*, 2013; Xia *et al*, 2008; Choi *et al.*, 2015b).

Animals administered zinc sulfate nanoparticles indicate better hematological and serum biochemistry value. These improved clinical lab values indicate lesser damage to the animals in-spite of greater zinc plasma concentration. These improved findings can be explain by the lesser/ negligible role of zinc sulfate in the production of oxidative stress as reported by various researchers (Ali *et al*, 2012; Heng *et al*, 2010; Kong *et al*, 2019; Setyawati *et al.*, 2013). Mild damaging behavior of higher dose of zinc sulfate nanoparticles is attributed to the zinc toxicity which is reported in large number of studies.

CONCLUSION

Significant improvement in the pharmacokinetics profile was evident. Moreover, greater safety profile and less toxic effects have been observed in animals given zinc sulfate nanoparticle. Furthermore, role of the zinc sulfate nanoparticles should be investigated in other pathological condition linked with oxidative stress like hepatotoxicity, nephrotoxicity, immunosuppression and etc.

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