

CYTOGENETIC AND SYSTEMIC TOXICITY INDUCED BY SILVER AND
COPPER(II) OXIDE NANOPARTICLES AND THEIR MIXTURE IN THE
SOMATIC CELLS OF THREE EUKARYOTIC ORGANISMS

BY

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ABSTRACT

Silver (Ag) and copper(II) oxide (CuO) nanoparticles are used in personal care products because of their antimicrobial properties. Their continual release into the environment may enhance genotoxic effects in the ecosystem, a condition widely reported from *in vitro* studies. However, *in vivo*, there is insufficient information on DNA and systemic damage, as well as the effect of the mixture of these nanoparticles in aquatic and terrestrial biota. This study was designed to investigate the genetic and systemic toxicity of Ag and CuO nanoparticles, singly and combined in somatic cells of three eukaryotic organisms and their mechanism of DNA damage.

The selected eukaryotic organisms were onion (*Allium cepa* Linnaeus), mud catfish (*Clarias gariepinus* Burchell) and mice (*Mus musculus* Linnaeus). Cytogenotoxicity of Ag, CuO and their mixture (1:1) was investigated at different concentrations using the *A. cepa* chromosome aberration assay (0, 5-80 mg/L; n=64), micronucleus assessment in peripheral blood of juvenile catfish (0, 6.25-100 mg/L; n=80) and bone marrow of male mice (0, 18.75-300 mg/kg; n=64). Haematological parameters [haemoglobin concentration, Packed Cell Volume (PCV), Red Blood Cell (RBC) and White Blood Cell (WBC) counts] were assessed in catfish and mice. The histopathology of their liver and fish gill was done using standard protocols. Mechanism of DNA damage was investigated by analysing hepatic oxidative stress biomarkers [Superoxide Dismutase (SOD), reduced Glutathione and Malondialdehyde] in both catfish and mice. Interaction Factor (IF) of the mixture was calculated according to standard method. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$.

In *A. cepa*, there was a concentration-dependent increase in the percentage frequency of dividing cells with Ag (1.3-1.6 fold); and decrease with CuO (1.1-16.8 fold) as well as mixture (1.5-2.7 fold). The frequency of aberrant chromosomes significantly increased only with Ag (3.3-8.7 fold) and mixture (1.5-4.6 fold) compared with control. Micronuclei induction with Ag, CuO and their mixture significantly increased in catfish (1.1-1.9, 1.4-2.2 and 1.6-2.9 fold), and mice (1.0-2.9, 1.1-4.8 and 1.5-3.1 fold), respectively. Haemoglobin concentration, PCV, RBC and WBC significantly decreased only in both nanoparticles and their mixture for catfish. Gill lamella hyperplasia and hepatocellular necrosis were observed in catfish and mice

respectively. In catfish, there were significant alterations in SOD activities (1.1-2.2 fold increase with Ag and CuO; and 1.6-2.0 fold decrease with mixture). Alongside, reduced Glutathione and Malondialdehyde levels (1.1-1.8; and 1.1-2.4 fold increase with Ag and CuO, respectively; and 1.1-2.8 fold decrease with mixture) were altered. In mice, there were significant alterations in SOD activities (1.1-1.6 fold decrease with Ag and CuO; and 1.3-1.6 fold increase with mixture), Malondialdehyde (1.1-1.5 fold increase with Ag and mixture; and 1.1-2.0 fold decrease with CuO) and reduced Glutathione levels (1.1-1.2 fold increase with Ag and decrease with CuO). The IF showed that interaction between Ag and CuO was antagonistic for cytogenotoxicity and oxidative stress.

Silver, copper(II) oxide and their mixture induced genomic disruption in the three organisms with systemic anomalies in *Clarias gariepinus* and *Mus musculus*. Oxidative stress in the exposed cells was responsible for the observed DNA damage.

Keywords: Metallic nanoparticles, Hepatic oxidative stress, DNA damage, Antagonistic interaction

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DEDICATION

This thesis is dedicated to my brothers; Oluseun, Mobolaji, and Olakunle for their unrelenting support towards the success of this Ph.D. programme.

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CERTIFICATION

I certify that this work was done by OGUNSUYI, Olusegun Ifeoluwa (159937) in the Cell Biology and Genetics Unit of the Department of Zoology, University of Ibadan, under my supervision.

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LIST OF ABBREVIATIONS

AgNPs:	Silver nanoparticles
ALT:	Alanine Aminotransferase
AST:	Aspartate aminotransferase
BSA:	Bovine Serum albumin
CA:	Chromosome aberration
CAT:	Catalase
CuONPs:	Copper(II) oxide nanoparticles
GSH:	Reduced glutathione
Hb:	Haemoglobin
MAPK:	Mitogen activated protein kinase
MCH:	Mean corpuscular haemoglobin
MCHC:	Mean corpuscular haemoglobin concentration
MCV:	Mean corpuscular volume
MDA:	Malondialdehyde
MI:	Mitotic Index
MN:	Micronucleus
MNNCE:	Micronucleated normochromatic erythrocytes
MNPCE:	Micronucleated polychromatic erythrocyte
NAs:	Nuclear abnormalities
NC:	Negative control
NCE:	Normochromatic erythrocyte
NF-kB:	Nuclear Factor kappa B
NMs:	Nanomaterials
NPs:	Nanoparticles
PCE:	Polychromatic erythrocyte
PCV:	Packed Cell Volume
RBC:	Red Blood Cell
RNS:	Reactive Nitrogen species
H ₂ O ₂ :	Hydrogen peroxide
O ₂ ^{·-} :	Superoxide anion
OH [·] :	Hydroxyl radical

NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
Nrf ₂ :	Nuclear Factor (erythroid-derived 2)-like 2
ROS:	Reactive Oxygen Species
SOD:	Superoxide Dismutase
WBC:	White blood cells

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CHAPTER ONE

INTRODUCTION

The nanotechnology industry is an exponentially expanding industry with myriad of promises and benefits that have significant impacts on the world economy, science and the society (Doak and Dusinska, 2017). The application of nanotechnology is evident in diverse areas such as environmental remediation, nanoelectronics, aerospace engineering, medical healthcare, household and consumer products. The success of the nanotechnology industry is attributed to the advent of nanoparticles which has unique and beneficial physicochemical properties which differs from those of their bulk counterparts (Kumar and Dhawan, 2013). These properties include higher chemical reactivity, improved thermal and /or electrical conductivity, catalytic and optical properties, high tensile strength and improved drug delivery capacity (Singh *et al.*, 2013; Kim and Hyeon, 2014). Reports indicate that 622 companies in 30 countries produce 1814 nanomaterial-containing products (Vance *et al.*, 2015). The revenue from nanomaterial- enhanced products increased by over 100 percent from \$ 339 billion to \$731 billion between 2010 and 2012 and its global value is expected to reach \$ 4.4 trillion dollars by the year 2018 (Lux Research, 2014). This dramatic expansion implies an increase in potential exposure to nanomaterials.

Nanomaterials (NM) or nano-objects as described by the International Organisation for Standardisation (ISO) (2008), are materials with one or more external dimensions which are equal to or less than 100 nm. Materials that possess one dimension at the nanoscale are referred to as nanoplates. Nanofibres possess two external dimensions and nanoparticles have three dimensions at the nanoscale level. Nanomaterial as an umbrella name consist of nanoparticles (NPs), nanotubes, nanofibres, composite materials and nanostructured surfaces. Nanoparticles that are intentionally produced in the manufacturing industries for a specific purpose and properties are referred to as engineered nanoparticles (ENPs) while those that occur incidentally from combustion and natural processes are referred to as ultrafine particles. Nanoparticles comprise metals (e.g. Ag, Au, Co, P, Ni), metal oxides (e.g. TiO₂, Al₂O₃, CuO, MnO₂),

fullerene, quantum dots carbon nanotubes and among others (Pattan and Kaul, 2014; Nam *et al.*, 2014). The fundamental unique property exhibited by NPs is their small size which allows possibility of its various functions and suitability. In other words, the nanoparticles have a larger surface area to volume ratio than their bulk form hence, a greater reactivity. Nanoparticles are integral constituents of toothpastes, room sprays, cosmetics, paper, plastics wares, electrical appliances, catalysts, lubricants, paints, drugs and medical equipment construction materials, optical devices, sensors, food packaging, fabrics, agrochemicals, water treatment, detergents, weapons/ explosives and other numerous products (Zhang *et al.*, 2015). In spite of the so many uses of NPs, there are scientific concerns that they may cause adverse health and environmental effects when they come in contact with biological systems (Cavallo *et al.*, 2012).

Human exposures to NPs may occur in the manufacturing, use and disposal process which could exist as aerosols, suspensions or emulsions (Kumar and Dhawan, 2013). Inhalation and dermal exposure have been described as the major routes of exposure in the workplace. Exposure can also occur through injection or medical implants in drug delivery and therapeutic applications (Ge *et al.*, 2014). Also, of importance is the oral route of exposure from food and food packaging as well as unintentional hand to mouth transfer during synthesis, transfer through the food chain from accidental discharge into the environment. In addition, NPs can be translocated from the mucocilliary system of the lungs following inhalation into the gastrointestinal tract (Sharma *et al.*, 2012).

The exponential increase in production as well as use of NPs and its related products will eventually lead to its discharge into the different ecological habitats around us. Hence, there is a risk of contamination of the various ecological media (air, soil, water) (Ghosh *et al.*, 2010). Evidence has shown that they end up in the aquifer from the disposal of items containing these nanomaterials (Kaegi *et al.*, 2010). Existing reports on the effect of nanoparticles release into the aquatic environment and organisms include behavioural alterations, biochemical responses, organ pathologies, oxidative stress and even mortality in some fishes (Choi *et al.*, 2010; Perera and Pathirante, 2012). Nanoparticles uptake by plants and aquatic animals may also end up being biomagnified in the food chain resulting in toxic reactions in different organ and body systems in higher organisms and humans.

Depending on size and aggregation of the NMs, they penetrate cells either by endocytosis through receptors or passive diffusion. When NPs pass across cell membranes, they interact with organelles in the cell. They can further bind to the nuclear material after their passage through nuclear pores or membrane. Binding can also occur after dissolution of membranes of the nucleus (if larger or aggregated) during cell division. Consequently, once inside the nucleus they can damage DNA through direct interactions with it or its associated histone proteins. This could also lead to disruptions of proteins involved in DNA transcriptions and replication (Cavallo *et al.*, 2012; Koedrith *et al.*, 2014). NPs can alter cell functions such as, metabolism, proliferation and death. Quite a number of diseases can have been associated with the alteration of these fundamental activities of the cells. These include neurodegenerative diseases and cancer. Production of ROS on nanoparticle surfaces is one of the possible molecular explanations for its toxicity at the cellular level which often results in inflammation, DNA modification, cell injury and apoptosis (Oberdörster *et al.*, 2005).

Various adverse health conditions such as asthma, emphysema, bronchitis, lung cancer, arteriosclerosis, arrhythmia and a few others have been associated with nanomaterials (Buzea *et al.*, 2007). Toxicological assessment of the nanomaterials widely in use or which may be applied in future is of importance as part of environmental risk assessment and management. of these materials which are already in use (Koedrith *et al.*, 2014)

Silver (Ag) and copper(II) oxide (CuO) NPs are examples of metal and metal oxide nanoparticles. They possess antimicrobial properties which makes them the nanoparticles of choice in numerous products. Silver nanoparticles which is also referred to as nanosilver constitute 24 % of all nanoparticles containing - products listed on the consumer product inventory (Vance *et al.*, 2015). They are used as coatings for hospital beds, medical implants, in textiles, wound and burn dressings, water purification chemicals, intragenital contraceptives air freshener, storage containers, room spray (Ge *et al.*, 2014). Although nanosilver has many uses in consumer products, the health effects of this material has not been well characterised *in vivo*, even though there are reports on *in vitro* toxicity of nanosilver (Powers *et al.* 2010). CuONPs have also found applications in biocides: face masks, socks, wound dressings materials; textiles, paints, plastics and food containers due to their

antimicrobial properties (Delgado *et al.*, 2011), in electronic chips, semiconductors, as well as in gas sensors, batteries, solar panels (Guo *et al.*, 2009); heat transfer nanofluids because of their brilliant thermal and physical characteristics (Ebrahimnia-Bajestan *et al.*, 2011).

There are pieces of evidence of contamination of surface water and the environment coupled with the rapid increase in production of NPs, but limited reports exist in literature on the genotoxic effects of AgNPs and CuONPs in plants and animals from aquatic and terrestrial environment. The toxicities of AgNPs and CuONPs have been explored in model systems such as mammalian cells and *in vivo* models (Chang *et al.*, 2012; Gosh *et al.*, 2012; Flower *et al.*, 2012; El Mahdy *et al.*, 2014). Studies on the toxicity of AgNPs have been carried out in mouse embryonic stem cells and fibroblast cells (Ahamed *et al.*, 2008), HeLa cell line (Miura and Shinohara, 2009), human liver cell (Piao *et al.*, 2011), rat liver cells (Teodoro *et al.*, 2011); testicular cells (Asare *et al.*, 2012), peripheral blood cells (Flower *et al.*, 2012), mouse bone marrow cells (animal model), *Allium cepa* and *Nicotiana tabacum* (Ghosh *et al.*, 2012). Toxicity of CuONPs in aquatic environment have also been reported in both invertebrates and vertebrates' systems (Kovriznych *et al.*, 2013); human lung cells (A549) (Ahamed *et al.*, 2010), human mesenchymal stem cells (Mancuso and Cao, 2014); cultured primary brain astrocytes (Buckle *et al.*, 2014), and human hepatocellular carcinoma cells (HepG2) (Siddiqui *et al.*, 2015).

Despite the various attempts to assess the toxicity of ENPs, there is still a large gap due to the complex nature of ENPs. Besides the size of ENPs, other physicochemical characteristics like the area of the NPs, crystal form, shape, surface charge and coatings aggregation, and presence of impurities which are not often reported are important factors responsible for the lag in advancement of nanotoxicology. Most of the existing studies are *in vitro* without corresponding *in vivo* assays. Unlike the *in vitro* assays, the *in vivo* assays provide insight on physiological factors such as metabolism, pharmacokinetics, DNA repair processes and other biological interactions within the body of an organism which may enhance or reduce toxicity. Furthermore, concentrations of NPs utilised at the *in vivo* study may differ from the *in vitro* study. Similarly, lack of proper characterisation of NPs makes it difficult to compare and assess the toxicity of NPs as well as lack of standard dosimetry for assessment of

nanotoxicity. As such, some of the studies were carried out at very high doses (Fabrega *et al.*, 2011; Reidy *et al.*, 2013).

1.1 Rationale/ Justification of the study

The ever-increasing rate of manufacture and application NPs, in numerous consumer, industrial and biomedical and products suggest and alarming concern for their safety for environmental and human health. Exposure to these NPs can lead to environmental contamination, undesirable health effects, in susceptible parts of the population and unforeseen deleterious consequences. Hence, there is need for timely assessment (Oberdörster, 2005; Koederith *et al.*, 2014).

Furthermore, existing studies on NM toxicity have been carried out but largely in *in vitro* systems with limited *in vivo* genotoxicity studies. Currently available data on AgNPs and CuONPs' cytogenotoxicity indicates potential DNA - damaging effects. However, *in vivo* investigations are limited and inconsistent (Klein and Godnic-cvar, 2012; Pattan and Kaul, 2014). Different routes of exposure have been characterised as one of the reasons for these inconsistencies. Others include size and structural characterisation of NM used in the studies (particle property and toxicity relationship). There is therefore a need for investigation of genotoxicity and its mechanism originating from intercellular processes in piscine and murine model via the oral route which is an important exposure routes to NPs from the environment.

In addition, there is insufficient information on potential *in vivo* cytogenotoxicity and mutagenicity assessment of AgNPs and CuONPs and no literature on their mixture in aquatic and terrestrial ecosystems. Also, assessment of the safe use of nanoparticle-containing products in this part of the world is crucial because, Nigeria as a nation imports so many products for use by her citizenry. Hence, there is risk of exposure to some of the nanoparticulate metals present in numerous consumer products.

1.2 Aim of the study

This study aims at investigating potential genetic and systemic toxicity of silver and copper(II) oxide nanoparticles and their 1:1 mixture using plant and animal models.

1.3 Objectives of the study

The objectives were to:

1. characterise the physicochemical properties of the silver and copper(II) oxide nanoparticles and their 1:1 mixture using transmission electron microscopy (TEM), zeta potential and dynamic light scattering (DLS) measurements
2. evaluate the genotoxic and recovery effects of exposure to the selected nanoparticles singly, and their 1:1 mixture using the *Allium cepa* chromosome aberration assay
3. determine the acute toxicity of silver, copper(II) oxide NPs in mud catfish (*Clarias gariepinus*) and mice (*Mus musculus*).
4. evaluate the genotoxicity of silver, copper(II) oxide NPs and their 1:1 mixture in *C. gariepinus* using peripheral blood micronucleus assay.
5. evaluate the genotoxic and recovery effect of silver, copper(II) oxide NPs and their 1:1 mixture using the bone marrow micronucleus assay in *Mus musculus*.
6. assess the effects of the nanoparticles and their 1:1 mixture on haematological indices and biochemical parameters of *C. gariepinus* and *Mus musculus*
7. assess the histopathological effect of the NPs and their 1:1 mixture on skin, gill, liver, and intestines of *C. gariepinus* as well as liver spleen and kidney of *Mus musculus*

1.4 Hypotheses of the study

Following the considerable increase in production and widespread use of nanomaterials in both industrial and household products, the following are hypothesized:

H₀₁: there is no significant genetic damage induced in *Allium cepa*, *C. gariepinus* and *Mus musculus* exposed to silver and copper(II) oxide nanoparticles and their 1:1 mixture compared to their corresponding controls.

H₀₂: There is no significant systemic toxicity induced in *C. gariepinus* and *Mus musculus* exposed to silver and copper (II) oxide nanoparticles, and their 1:1 mixture when compared with unexposed organism.

H₀₃: There is no significant difference in genetic damage induced by silver, copper(II) oxide nanoparticles and their 1:1 mixture within the exposure period and recovery period in *Allium cepa*, and *Mus musculus*.

1.5 Importance of the study

It is expected that this study will answer questions on *in vivo* cytogenotoxicity and mechanism of genotoxicity of AgNPs, CuONPs, and their 1:1 mixture. Findings will provide information on nano genotoxicity of the two NPs. It will reveal potential human and environmental health risk that may arise from exposure to these NPs and their co-exposure. Furthermore, information from this study will add tremendously to existing knowledge needed for the formulation of policies and framework for regulation of the manufacture and use of nanomaterials. In addition, it will enlighten the populace about nanoparticles and the potential risks of their indiscriminate use and disposal.

CHAPTER TWO

LITERATURE REVIEW

2.1 Nanotechnology

Nanotechnology is a science field that involves other disciplines and it has witnessed exponential growth due to its practical use in a lot of industrial, medical and household products (George *et al.*, 2017). It involves the processes of designing, synthesising, and production of devices and materials at the nanoscale. (Lux Research, 2014; Koedrith *et al.*, 2014). The rapid growth of the nanotechnology industry is as a result of its scientific and economic benefits accrued from the design and production of nanoparticles with distinct physicochemical properties. These include improvement in optical features, sensitivity, and long-lasting properties of nano – enabled products.

Currently, almost a thousand products made up of nanomaterials have been identified and sales value of over three trillion US dollars as at 2015. Report indicates that commercial nanotechnology industry revenue (of nano-enabled products) increased by over 100 percent from \$339 billion in the year 2010 to \$731 billion in 2012 and currently, the global value of engineered nanomaterials, nanodevices and products is expected to reach \$4.4 trillion dollars by the year 2018 (Lux Research, 2014). Therefore, human and environmental health consequences from the exposures to nanoparticles are of special interest to scientists. The safety of nanotechnology products has become a global issue that concerns scientist, regulators, industries and the public at large (Kwon *et al.*, 2014).

2.1.1 Applications of Nanotechnology

Nanotechnology has become part of our everyday lives and is of immense benefits in many sectors, it is helping to revolutionize, numerous sectors at a phenomenal rate. They are used in consumer products for food packaging because of its antibacterial, antifungal properties and smart packaging; sports and leisure household use in tennis racket, nanoceramics for high strength; textile and clothing for provision of stain resistance, waterproof, antibacterial, UV protection and many other desirable properties; computing and electronics to offer faster based chipsets, memory and processors; Transport building and construction are not exempted as they are applicable in the production of paints with improved adhesion and anti – mildew properties, transport lighter materials with more efficient fuels, self-cleaning glass sheets etc. In addition, medical uses in diagnostic devices, targeting and localized delivery of drugs have also been reported. Furthermore, nanotechnology is applied in environmental remediation for safer and more efficient waste management and water purification in the reduction of pollution (Vivek *et al.*, 2009; Roco, 2011).

2.2 Nanomaterials

Nanomaterial (NM) is an umbrella term nanosized morphologies with more than one of dimensions within one and a hundred nanometre range; these includes nanoplates, nanofibres, nanotubes, and nanoparticles (NIOSH, 2009; Ng *et al.*, 2010). Nanoplate refers to materials with only one of its external dimensions at the nanoscale; nanofibre has two of its external dimensions at the nano level while the nanotube is characterised as a nano structure with hollow while nanorod is a solid structure; and nanoparticles, have all its 3 dimensions at the nanoscale. NMs when suspended in a gaseous state are referred to as nano aerosol. They can also be found in a liquid suspension (often referred to as colloid or nanohydrosol), or in a matrix which is referred to as a nanocomposite (NIOSH, 2009).

2.3 Nanoparticles

There are different definitions of NPs. The European Union (2011) defines it as “particles in unbound state, agglomerate or aggregate from where 50% or more of the particles exhibit one or more external dimensions in the size range 1 – 100 nm” However, the most widely used definition is a particle possessing one or more of

its dimension below 100 nm range (Magdolenova *et al.*, 2014). A nanosize material (< 100 nm) is smaller than a red blood cell (Figure 2.1) which is in micron size range.

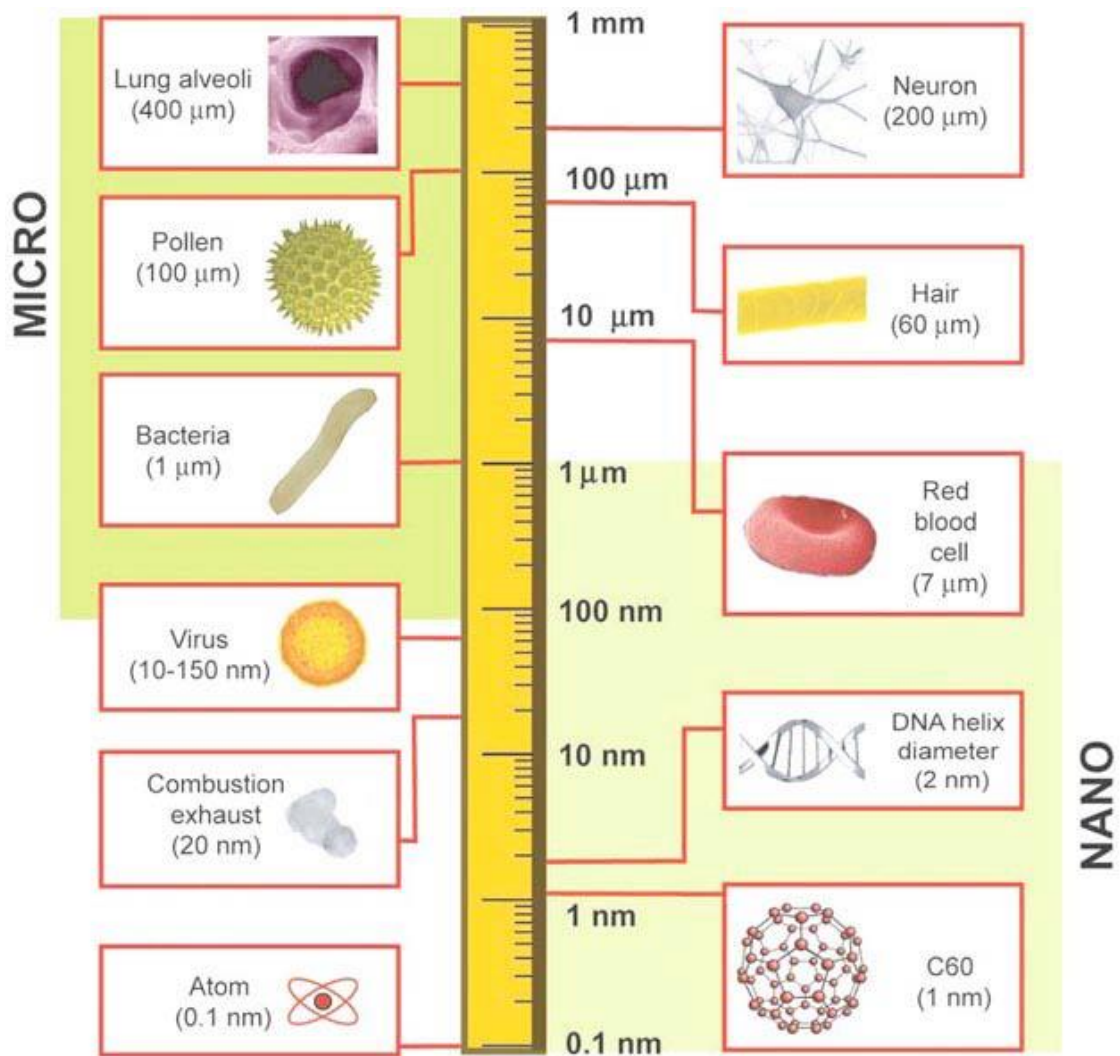


Figure 2.1 Comparison of different sizes of biological components/ organisms to nanomaterials

Source: Buzea *et al.* (2007)

2.3.1 Types of nanoparticles

Nanoparticles can be classified based on occurrence. NPs that exist naturally are referred to as naturally occurring nanoparticles (NNPs). Examples of such are those produced in volcanoes, forest fires, storm dust. Also, nanoparticles are characterised as anthropogenic nanoparticles which occur as either unintentional or deliberate actions of humans as the name implies. Examples of non – intentional NPs includes by-products of combustion of domestic sewage sludge, coal and fuel, cigarettes smokes, cooking, manufacture of chemicals, welding, smelting, ore refining and automobile combustions, while deliberately produced NPs are the engineered NPs (ENPs). They are fabricated for specific purposes and they include nanoparticulate forms of carbon and inorganic chemicals. These includes nanoparticulate forms of metal (e.g. copper, silver, iron, gold); metal oxide (e.g. ZnO, CuO, MnO₂, Al₂O₃, CeO₂); the carbon based (like the multi and single-walled types); nanowires; the quantum dots and fullerene derivatives (Ng *et al.*, 2010). Table 2.1 highlights some ENPs and their applications.

2.3.2 Unique properties of NPs

Size: NPs size is its foundational unique property. The tiny size of nanoparticles (< 100 nm) provides it with very efficient surface, and more atoms on the particle surfaces compared to their bulk forms. As a result of this, NPs exhibit special properties (e.g. magnetic, optical, electrical and catalytic properties) (Dusinska *et al.*, 2009; Kwon *et al.*, 2014).

Optical features: colour change is often observed as the size of metals transits from bulk size to nanosize. This unique property of NPs is associated with their power to restrict electrons to a very small size thereby generating quantum effects. Silver nanoparticle, for instance, changes colour from yellow when in suspension to blue color in clustered form.

Table 2.1. Some applications of nanoparticles

Nanoparticles or nanomaterial	Application
Aluminium oxide	Optical polishing, cosmetics, and clothing
C ₆₀ fullerenes	Hydrogen storage, drug delivery, therapeutics, coatings and pigments, lubrication and cosmetics
Carbon nanotubes (single or multi walled)	Hydrogen storage, drug delivery, textiles, electronics, water purification and sporting equipment
Ceramics	Electronics, anti – oxidants, and car polish
Copper or copper oxide	Lubrication oil additive, electronics and computer processors, conductive coatings, printer ink, sintering additives, anti – ageing cream and skin conditioner, and mineral supplements
Gold	Drug delivery, labels of immunocytochemistry, and biological hazard detection e.g. ricin, <i>E. coli</i> , and mineral supplements
Iron oxide	Ultrafiltration and oxidation reduction catalyst
Iron sulphide	Removal of organochlorine pesticide from drinking water
Nanocrystal	Insulators and drug delivery
Nano – rods	Electronics, sensors and sensing devices
Polymers	Therapeutics, coatings and pigments, lubrication, absorbents
Quantum dots	Medical imaging
Silica	Photovoltaics, optics and optical devices, anti – graffiti paints and cosmetics
Silver	Antibacterial uses in water treatment, fabrics softener, clothing, soft toys, wound dressing, kitchen utensils and appliances, computer keyboards, food storage containers, and baby products (e.g. cup and feeding bottles) and uses in contraception and toothpaste
Titanium dioxide	Paint, sunscreen, cosmetics, capacitors, building materials, catalyst, air clearance, anti-bacterial, viral, algal, fungus and mould coating for domestic baths, and sporting equipment
Nano – vitamins (some vitamins encapsulated in nano – delivery vehicles)	Vitamin E; food, beverages and cosmetics, vitamins B12 and E; cosmetics
Zinc oxide	Sunscreen, cosmetics, cosmetic remover, foot deodorant and car polish

Source: Shaw and Handy (2011)

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similarly, the colour of gold NPs switches from blue to magenta, as its size changes (Kwon *et al.*, 2014).

Chemical reactivity: NPs show higher chemical reactivity due to quantum and surface effects. (Roduner, 2006; Buzea *et al.*, 2007). The proportion of the atoms on the surfaces of NPs are enormous in comparison to their bulk counterpart. In other words, nanoparticles possess large particle number per unit mass. A comparative assessment shows that a carbon microparticle that is 60 μm wide with 0.3 μg mass will have a surface area of 0.01 mm^2 . While, its nanoparticulate form, of sixty nanometer diameters, possess a larger area of 11.3 mm^2 and 1 billion nanoparticles. Chemical reactivity increases with decrease in nanometer size. However, coatings on it could affect the particles reactivity and possibly reduce (Roduner, 2006).

Melting point: This is usually lower than that of their macro sized counterpart. because at the nanoparticulate level, their surface atoms have fewer neighbors than those of the bulk counterpart. Hence, this culminates into a lower binding energy. A practical example is the lower melting point exhibited by the gold nanoparticles. The melting point of a 3 nm gold nanoparticles is far lower by 300 degrees compared to that of bulk gold (Roduner, 2006).

Electrical conductivity and Magnetic properties: the quantum effect play determines the electronic and magnetic properties of NPs. The spatial arrangement of atoms for example in quantum dots in the three spatial direction confers higher electrical conductivity. Also, the numerous unpaired electrons make the nanoparticulate forms a better magnetic element in comparison with the bulk form. This enhances either acceptance or donation of charge (Roduner, 2006).

Suspension Formation of NPs in media: forces of interaction between nanoparticles and suspension media often quite enormous such that they form suspension. On the other hand, the bulk form either sink or float in a liquid media the density gradient of the material and the liquid media. (Kumar *et al.*, 2012). However, NPs disperse in aqueous media, as a result of their steric and electrostatic repulsive force (Maynard, 2007).

Other unique properties of some NPs include surface plasmon resonance, better malleability and ductility as observed in doped silicon nanocrystals, ZnO, and copper (Rowe *et al.*, 2013)

2.4 Nanoparticles exposure and release

Man is at risk of exposure to NPs when it is being manufactured, utilised or disposed. Main routes of exposure are inhalation, oral and dermal route via lungs, mouth, and skin respectively. Other potential exposure routes include intraperitoneal, intravenous and intradermal routes (Yah, *et al.*, 2012; Kim *et al.*, 2013b). An organism may come in contact with NPs via the three major different exposure routes depending on the use of NPs and habitat where the organism lives. For instance, in aquatic environment exposure of the organisms occurs oral, inhalation and dermal. Continuous and increased exposure in such an environment could lead to bioaccumulation. The transfer of the accumulated NPs in the organism from prey to predator is a possibility and could result in biomagnification. The ecotoxicological studies on known pollutants have shown that biomagnification of pollutant is dangerous to the public health (Dalai *et al.*, 2014).

There is a rapid increase in the release of nanomaterials into the surrounding ecological media as a result of the numerous advancements in application of nanomaterials in diverse areas (Gupta *et al.*, 2016). Reports of Keller *et al.* (2013) and Keller and Lazareva (2014) indicated that the major sources of release of nanomaterials into the environment during use include paint, cosmetics, pigments, and coatings. Furthermore, nanomaterials released into the environment are often detected in sewage treatment plants and aquatic environment which are ultimately their sinks. In addition, the authors noted that a huge volume of nanomaterials as high as 69,200 and 189,200 metric tons are released globally and annually into water and landfills, respectively.

Nanomaterials enter into the surrounding air, soil or water at different phase of utilization (Figure 2.2), these includes transportation, research and development, fabrication, (Gupta *et al.*, 2017). On entering into the environment, they move across different media and interacting with the entities in the environment (physical, chemical and biological) which can curtail or inhibit their behaviour and transport in the

ecological air, soil and water. The presence of nanomaterials in the environment may lead to interaction with various organisms in the food chain. The means by which an

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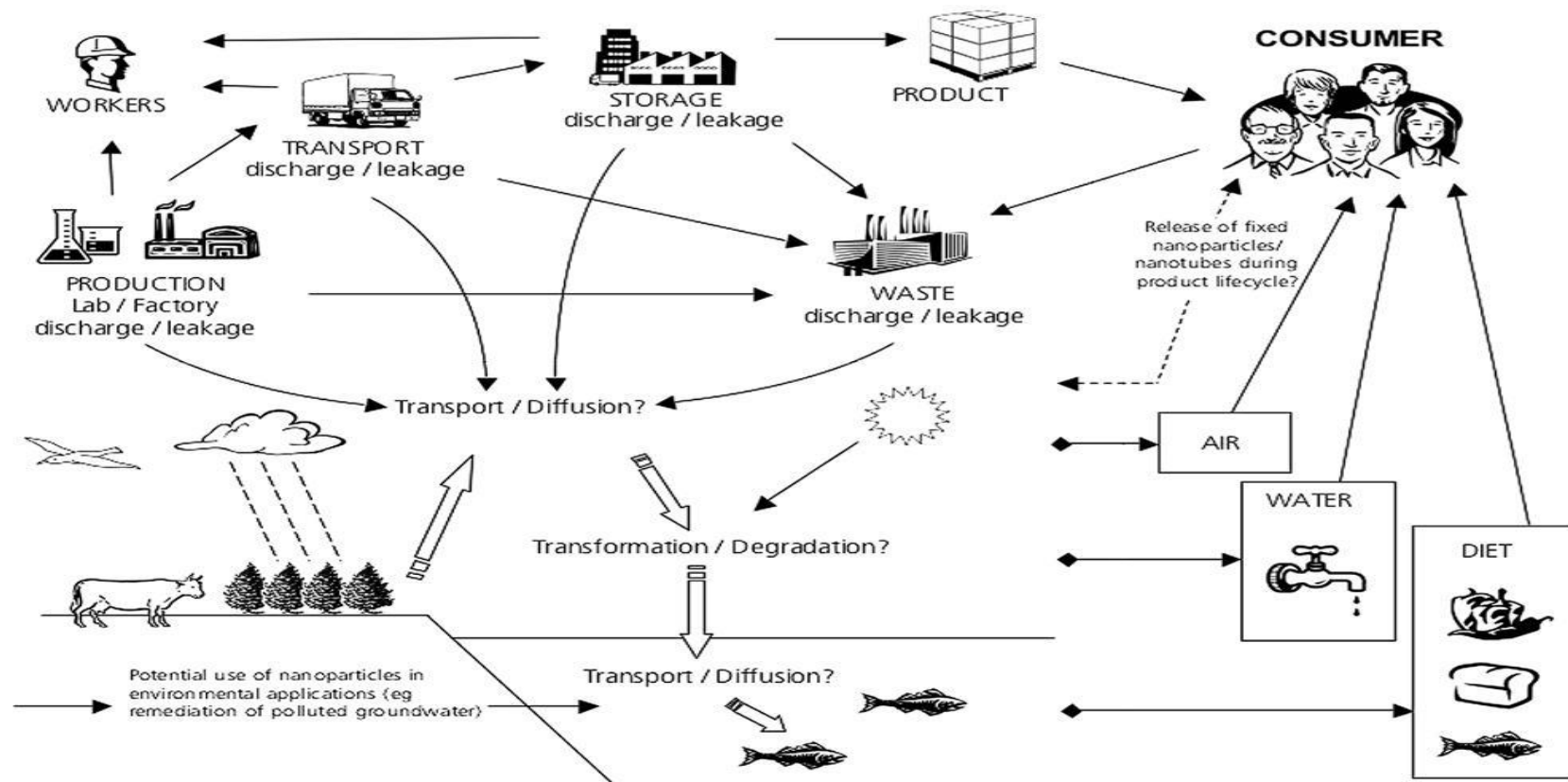


Figure 2.2. Possible release and exposure routes of NPs during manufacture and use in terrestrial and aquatic environments.

Source:

The

Royal

Society

(2004)

organism comes in contact with NPs dictates the rate at which the NPs is taken up into the organism and ultimately the potential adverse effect (Gupta *et al.*, 2017). Currently, there is scarce information in literatures on the environmental concentration of the nanoparticles especially in Africa but low concentration of engineered nanomaterials has been predicted in the natural water bodies. According to Gottschalk *et al.* (2015), the predicted concentration of nTiO₂, nZnO, and nAg are 0.6–100, 0.09–13 and 0–0.044 ng/L respectively. However, with the exponential rise in figures and magnitude of applications, it is anticipated to increase in the nearest future because of lack of proper disposal and necessary waste management practices. These necessitate the urgent need for safety assessment of ENMs for potential environmental health hazard and the sustainability of the nanotechnology industry.

The release of metal and other dust NPs occurs in the air from workplace and use of consumer products like sprays, air freshener, incidental activities and natural disasters. The inhalation of metallic or other dust has potential negative health effects. Different types of lung diseases due to inhalation of dust have been identified. The occurrence of these conditions depends largely on the type of the NP, dose and duration of exposure. Reports have shown that nasal exposure of the fumes of some metals (like copper) might result to fume fever, which is characterised by an influenza-like reaction. Also, metal dust such as cobalt, platinum, chromium and nickel, can result into asthma. Possible complications from inhalation of other metal fumes include fibrosis, and ultimately cancer of the lung. About 15 percent of lung cancer cases have been associated with occupational hazard as well as exposure to metals being a major cause (Buzea *et al.*, 2007).

2.5 Toxicokinetics of Nanoparticles

Toxicokinetics of NPs refers to the rate at which NPs gain entry into the biological system (which can occur via several routes) and it what happens to it following entrance into the body (Shi *et al.*, 2013). This depends on the rate at which the nanoparticle is taken up by the cell (absorption), distributed, metabolized and possibly passed out as faeces or urine. The exposure route determines how NPs migrate within the body system i.e. how it is translocated to the distal/ target organs (Figure 2.2). For instance, inhaled nanoparticles are deposited at different sites in the respiratory tract such as nose,

pharynx, lungs. Majority of inhaled particles end up in the lung. However, absorbance depends on the size of the nanoparticle. For example, a particle with a diameter < ten microns can get to the alveoli while much bigger sizes will be trapped down in the upper section of the lungs due to the force of gravity and interception (Buzea *et al.*, 2007).

Clearance of NPs deposited at the upper respiratory tract involves mucociliary escalators and is determined to a great extent by solubility, size, and concentration of nanomaterial. The elimination process is slower for insoluble nanoparticles compared to soluble nanoparticles. These trapped NPs eventually end up in the end up in the gastrointestinal tract. On the other hand, macrophages are employed to remove the particles from alveoli to the mucocilliary at the lower airways. Reports have shown that nanoparticles were translocated across the epithelial layer of the respiratory tract following deposition in the lungs into lymphatic vessels and circulatory system (Geiser *et al.*, 2005). The NPs are then transferred from the systemic circulations to the heart, kidney, liver, spleen, bladder and even the bone marrow (Oberdörster *et al.*, 2005)

Oral exposure to nanoparticles through food and food packaging, drugs, water, or cosmetics, dental prosthesis debris also find their way into the bloodstream after passing through a complex barrier-exchange system. The epithelial layer of the intestinal walls is in proximity with the ingested material, and absorbs ingested materials by the villi. The level of the NPs absorption in the gastrointestinal tract is influenced by the NPs physical and chemical properties, exposure or administration period and dosage. When nanoparticles, are absorbed into the biological system through oral route, they are distributed into various body organs/tissue, such as the stomach, spleen, liver, kidney, among other organs (Park *et al.*, 2010a).

Also, dermal uptake of nanoparticles passes through skin penetration barriers. The skin comprises 3 layers which are the subcutaneous (innermost layer), dermis (middle layer) and epidermis the uppermost layer. The uppermost part of the epidermis is a ten-micrometer thick layer containing keratin. This blocks the passage of substances. The epidermal surface consists of numerous microstructures, with a scale – like surface, sweat pores, sebaceous glands, and follicular hair spaces. They are capable of getting into the deep skin layers (Orbedoster *et al.*, 2005). Possible means by which

NPs penetrate the skin include hair follicles or broken skin. This is then further internalized by keratinocytes into cytoplasmic vacuoles and may consequently stimulate the production of pro-inflammatory mediators. The dermis is highly vascularised with macrophages, dendritic cells, neurons and lymph vessels. Hence, presence of nanoparticles in the epidermis and dermis are fully recognized by the immune system (Orbedoster *et al.*, 2005). Irrespective of exposure route, once the nanoparticles have reached the systemic circulation some proportions are liable to excretion or clearance in urine or faeces through the kidney or bile (Shi *et al.*, 2013).

2.6 Some factors affecting the toxicity of NPs

The physicochemical characteristics of nanoparticles (Figure 2.3) affects their toxicity and genotoxicity to living systems. NPs' properties like its morphology, surface composition and properties, solubility, aggregation/agglomeration, NP cell penetration, cell type, toxins and heavy metals present as impurity alongside the NPs, often contribute to their toxic or genotoxic effects. Physicochemical properties of NPs determine its biological activity and potential toxicity (Chan 2006; Vega-Villa *et al.* 2008). Therefore, Warheit (2008) suggests the proper characterisation of NPs' size, surface area (dry state), distribution (wet state) in appropriate culture medium which are similar to fluids in the biological system, crystallinity, aggregation pattern in properties, its contents, materials on its surface, reactivity, purity and synthesis method of NPs.

2.6.1 Particle size and shape

The nanosized nature provides greater reaction surface compared with the bulk counterpart. This enables nanoparticles to penetrate through membranes into the biological system. They move across the intestinal wall into the systemic circulation and thereby penetrating various organs and tissues. The size of NPs also determines rates of cellular uptake which in turn determines toxicity. Chang *et al.* (2012) showed that NPs with much lower sizes go as far as deep into the lungs and thereby causing more toxic effect than the larger sizes. In a study, Gurr and colleagues. (2005) examined the role of NPs' size on genotoxicity using different size of TiO₂ NPs (10, 20, 200 and > 200 nm) The authors showed that smaller sized TiO₂ (10, 20 nm) had higher genotoxic effect compared with TiO₂ of higher sizes (200 nm above). Haase *et*

al. (2011) also reported findings close to the theirs. They also discovered from a comparison of different sized AgNPs that smaller sized NPs induced higher levels of cytotoxicity in

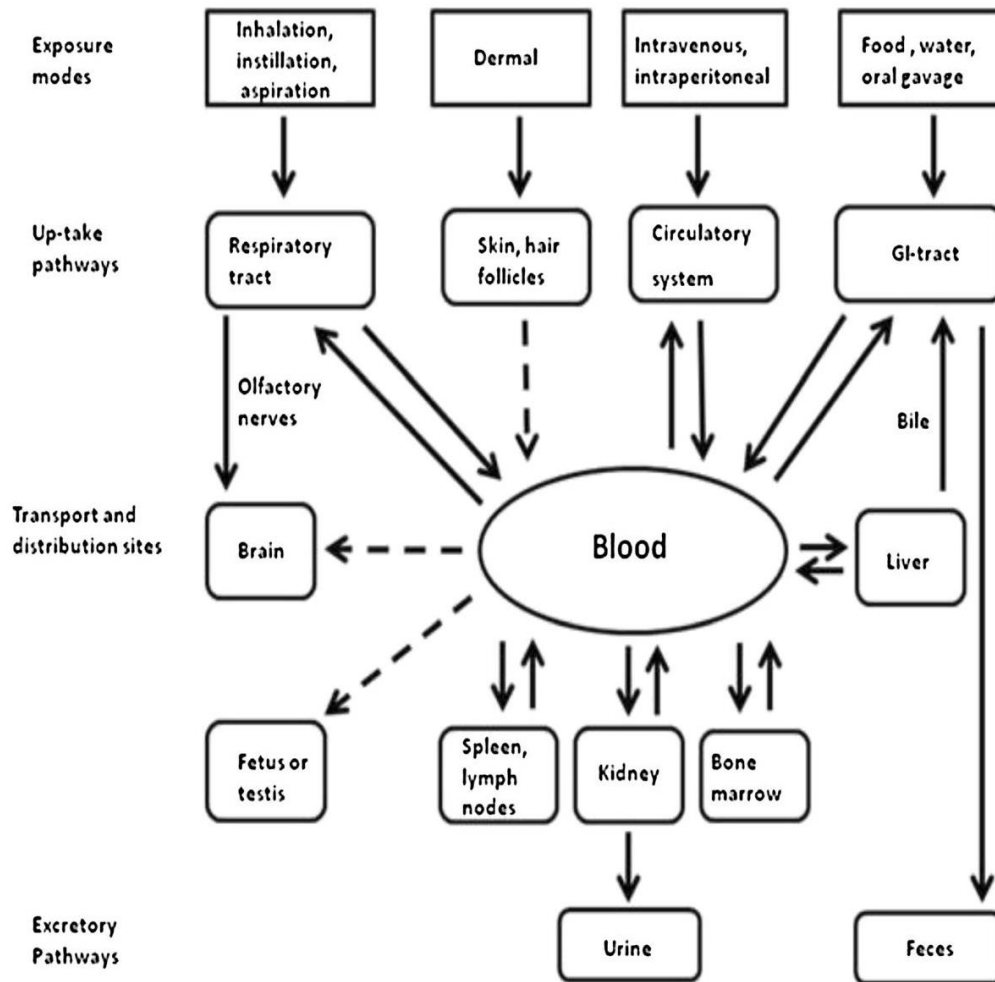


Figure 2.3. Toxicokinetics pattern of nanoparticles in biological system

NB: dotted lines refer to routes that are not certain

Source: Shi *et al.* (2013)

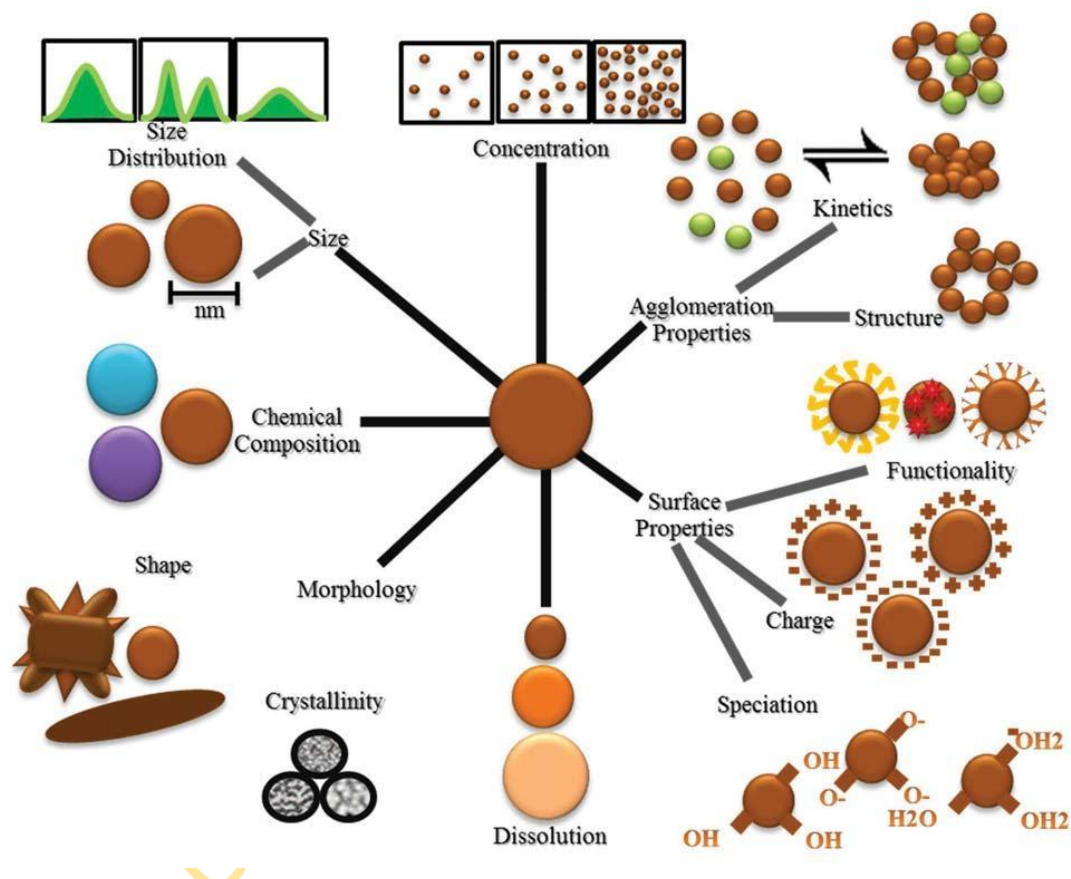


Figure 2.4. Diagrammatic representation of important physicochemical properties that affects NPs toxicity in aqueous media.

Source: Koedrith *et al.* (2014)

comparison to the larger forms. Also, investigations of Butler *et al.* (2015) revealed that higher levels of toxicity in Jurkat and THP -1, and *Glyptotendipes tokunaga* cells exposed to small AgNPs than to larger AgNPs which strongly indicates a key role of NPs' size in its rate of cellular uptake (Choi *et al.*, 2015).

In addition, to size, morphology also contribute to NPs behaviour in terms of aggregation kinetics and consequently affecting their distribution and bioavailability in the environment. Reports on the effect of shapes of silver and copper(II) oxide revealed that silver nanoparticles affect toxicity in bacteria and copper(II) oxides (Siddiqui, *et al.*,2015). A report on titanium dioxide genotoxicity proved that the different polymorph (anatase, brookite, and rutile) shows different reactivity. The anatase inducing higher genotoxicity in comparison with rutile (Sayes *et al.*, 2006; Iavicoli *et al.*, 2011).

2.6.2 Surface properties/Chemical composition

Surface properties such as the surface area charge (zeta potentials), speciation, functionalization as well as its hydrophobicity all contributes significantly to NPs toxicity. The Surface areas is determined by NPs' size and morphology. As the NPs size decreases and surfaces area increase so also reactivity of NPs increases. The surface charge explains the movement of NPs in an aqueous medium. And provides information about its stability. Charges higher than ± 30 mV are considered stable. In addition, the oxidation state of the nanoparticle (surface speciation) vary with time and therefore will determine the fate and behaviour nanoparticle in biological media and living systems. Often times, nanoparticles are functionalized with a range of molecular groups to enhance its properties. This also affects the aggregation and charge of NPs. The capping agent may affect its stabilization and reactivity. Functionalization of NPs enhances its dispersion properties in solution; increases solubility in some cases and overall it may also prevent the loss of the majority of the size-dependent effects (Koedrith *et al.*, 2014).

Park and colleagues (2013) observed that surface charge affects toxicity. Their study revealed that silica nanoparticles size and surface charge affected its toxicity in human keratinocytes; negatively charged silica nanoparticles were more toxic compared to the weak negatively charged NPs. Hu *et al.* (2009) also presented findings in their study which shows the dependence of metal oxide NPs' cytotoxicity on surface charge. The NPs toxicity to bacterium decreased with increase in cation charge increased (Kim *et al.*, 2014).

De Berardis *et al.* (2010) identified that chemical composition is a factor affecting NPs toxicity. The authors observed different effects in marine algae when exposed to ZnO and TiO₂ NPs, indicating importance of NPs composition.

2.6.3 Dissolution

Dissolution is the phenomenon whereby solid dissolves in a solvent medium. Scientific studies have highlighted that larger particles are impermeable into the cell walls of plant leaves and roots while soluble metal ions were absorbed (Proseus *et al.*, 2005; Asli *et al.*, 2009). The authors pointed out dissolution as an important reason for toxic effects of NPs observed in many organisms. Nanoparticles possess large surface area which enables high level of interaction with solvent molecules. Blinova *et al.* (2010) suggested dissolution order of CuONPs, which is far higher compared to that of bulk CuO could be responsible for its toxicity. However, some studies have disputed the role of solubility on nanotoxicity stating that metal ion dissolution plays minimal role in NPs toxicity (Midander *et al.*, 2009; Moos *et al.*, 2010).

2.6.4 Exposure Routes

The route of exposure is another fundamental factor that can lead to varying toxic effects. The Toxicokinetics of NPs from different exposure routes will determine the absorption rate, retention, metabolism and accumulation as well as clearance from target sites. Different route of exposure to NPs include intratracheal (pulmonary toxicity), oral, nasal, skin, intraperitoneal routes. (Chang *et al.*, 2008).

2.6.5 Dispersion media / Preparation of the NPs

The properties of the solvent (e.g hardness, pH, temperature salinity and impurities or dissolve organic particles) are capable of influencing the toxicity in the biological system (Handy *et al.* 2008). Therefore, NPs exhibit different behaviours in different types solvents. The type of solvent employed will determine cellular uptake and localisation of the NPs. Vevers and Jha (2008) observed different effects of well-characterised titanium dioxide on fish cells *in vitro* when dispersed in tissue culture media, PBS and water. There is a tendency that NPs will be form protein corona from its combination with the surrounding protein which influences genotoxicity and aids dispersion of NP (Gonzalez *et al.*, 2010).

2.6.6 Concentration of NPs

Due to Van der Waal forces, NPs often agglomerate both in suspension and dry form. This can have an effect on the stability of NP suspension over time. More so, there is a higher tendency of agglomeration at high concentration (Wang *et al.*, 2012b).

2.6.7 Impurities, physical and chemical agents

Presence of impurities in NPs as well as physical and chemical agents also affect toxicity. Agents like UV irradiation or chemical (polycyclic and aromatic hydrocarbons) under which organism is exposed are very important (Vevers and Jha, 2008). Some NPs (e.g. ZnO NPs) behave differently in the presence of radiation. also observed the effect of impurities in AuNPs stabilized by citrate ions. The authors reported that pure AuNPs did not induce mutagenicity under ordinary condition but caused damage in the presence of impurities (Wang *et al.*, 2012b).

2.6.8 Cell type

Dusinska *et al.* (2011) reported that different cell types / target cells behave differently with same NPs. Due to varying metabolic activities, different toxicological responses are often elicited by different types of cells (Vevers and Jha, 2008). Other factors such as the varying types of cell surface receptors enzymes, hormones, antioxidant activities and DNA repair efficiencies can also affect kind of toxic effect observed in different

cell types. In addition, different rates of NPs internalization, phagocytosis, and cytoplasmic inclusion are also important (Magdolenova *et al.*, 2014).

2.6.9 Assay type, conditions and models used

Method of evaluation of toxicity or genotoxicity varies with different endpoints. Therefore, different findings may occur. For instance, some assays detect DNA damage (e.g. comet assay) while another one might be more sensitive/ appropriate in the measurement of gene mutations (e.g. the *HPRT* assay), chromosomal abnormalities (e.g. chromosomal aberration or micronucleus assays). Also, comparison of results from *in vivo* test with *in vitro* assays is limited to primary genotoxicity but with *in vivo* tests, secondary genotoxicity arising from production of ROS by inflammatory cells and other pharmacokinetic interaction takes place (Magdolenova *et al.*, 2014). Toxic consequences of NPs also vary based on the type of cells used or biological model for the study as well as the condition of exposure

2.7 Silver nanoparticles (AgNPs)

It's soft, whitish, lustrous transition element that possess a very high thermal and electrical properties. The discovery of silver dates back to ancient Greece and Egypt times when it was a component of water storage vials to keeping water fresh. During the world war, it served purposes of prevention and treatment of infections (Bondarenko *et al.*, 2013; Firdhouse and Lalitha, 2015). Other uses include ointments, coins, foils, vessels, sutures, and colloids as lotions. Chemical properties of silver includes: Atomic number -47; Molecular weight -107.8682g/mol; Natural isotopes $^{106.90}\text{Ag}$ and $^{108.90}\text{Ag}$ with abundance in 52% and 48% respectively; Standard state –solid at 298° k; Crystal structure of silver – face-centered cubic; Melting point -961.93° C; Density -10.5g/cm³; Boiling point -2,212.0° C.; High thermal and electrical conductivity; Solubility in water - insoluble in cold or hot water.

2.7.1 Applications of silver nanoparticles

Silver as a metal has been grouped into the class of environmentally hazardous chemicals by the EPA as a result of its toxicity to aquatic plants and animals (Patlolla *et al.*, 2012). However, with the advent of nanomaterials with unique properties and

perceived toxic free nature attention is being shifted to use of silver nanoparticle. Today, silver nanoparticle (< 100 nm) is rapidly been utilised in the manufacture of numerous products due to its long-standing antimicrobial properties. Over 30% of products registered in nanoproducts database contains nanosilver. Moreover, in quite a number of products, silver nanoparticles are added to other materials to enhance the product quality (Reidy *et al.*, 2013; Vance *et al.*, 2015). It is an effective biocide with effectiveness against a wide variety of bacteria, component of wound dressings and catheters, therapeutic activities or a number of illnesses (e.g. cancer, lupus, tuberculosis, tetanus, typhoid). It is found in products such as textiles (T – shirts, socks, underwear, sports clothing etc), cosmetics, deodorant, bandages, contraceptives, sport materials, food packaging, detergents, female hygiene products, coatings of refrigerators, vacuum cleaners, dental materials, sterilizing agents in hospitals, paints, home appliances (such as automatic washer, filters), automotive upholstery, shoe soles, brooms (Patlolla *et al.*, 2015a).

Also, it is used in imaging and bio – sensing equipment because of its special optical light scattering and plasmon-resonance properties; in a number of assays as tags for quantitative detection of substances in biological systems. The fact that they do not undergo photobleaching like most fluorescent dyes. Silver nanoparticles are frequently used in polymeric, colloidal, spun and powder forms (Shoultz – Wilson *et al.*, 2010). Also, it is applied in the production of composite structures and inks, glues, pastes, polymers, thinner coatings etc (Ahamed *et al.*, 2010).

2.7.2 Synthesis of silver nanoparticles

Synthesis methods include top-down and bottom-up approach using either chemical, physical, or biological means. Different methods are often used in the synthesis of silver nanoparticles. These includes electrochemical reduction, microwave-assisted synthesis, ultrasonic-assisted reduction e.t.c. The top-down techniques involve the mechanical reduction of silver metal in its bulk form to the nanoscale through specialised methodologies like laser ablation and lithography while the bottom-up techniques entails chemical production process which makes use of a silver salt in

solvent and the reduction of the salt using a reducing agent accompanied with stabilizing agents to prevent formation of agglomerates (Firdhouse and Lalitha, 2015).

Silver nanoparticles can be synthesized using bacteria, fungi, and plant extracts; the biosynthetic procedure involves the use of microorganisms that have the ability to reduce silver salts (Kaluza *et al.*, 2009). It is an approach involving oxidation/reduction processes; metallic salt is being acted upon by enzymes produced by microorganisms and the chemical products of plants to produce nanoparticle. One distinct merit of this method is environmental friendliness (Prabhu and Poulouse, 2012).

2.7.3 Toxicity of silver nanoparticle

2.7.3.1 *In vitro* toxicity

Various reports from *in vitro* assays have shown toxic effect and oxidative stress, in different cell exposed to silver nanoparticles. Cytotoxicity of AgNPs was reported in human cell lines of different origin such as the liver, skin, lung, blood, cervix (Foldbjerg *et al.*, 2009; Samberg *et al.*, 2010) and murine macrophage cell, liver (Park *et al.*, 2010b), Chinese Hamster ovary cell (Souza *et al.*, 2016).

Reports of Hussain *et al.* (2005) showed significant impairment in hepatic mitochondria following exposure to silver nanoparticles (15 and 100 nm) at dose range of 5 to 50 $\mu\text{g/mL}$. Similarly, Burd *et al.* (2007) showed that AgNPs also impaired mitochondria function in HEKs and fibroblasts cells in the presence of AgNPs at 15 $\mu\text{g/mL}^{-1}$. Arora *et al.* (2009) also observed that AgNPs (7- 20 nm) caused apoptosis and damage to mitochondria in the carcinoma cells from the human skin following treatment with at 0.78 $\mu\text{g/mL}^{-1}$ and 1.56 $\mu\text{g/mL}^{-1}$.

Braydich-Stolle and *et al.* (2005) reported the potential ability of AgNPs (15 nm) to induce leakage of LDH and cause a decrease in the functional capacity of mitochondrial in C18-4 spermatogonial cell lines at concentrations above 5 $\mu\text{g/mL}$. Reports have also shown that AgNPs (25 nm) perturbed mitochondrial function and increased the generation of ROS in neuroblastoma cells from mice at 25 $\mu\text{g/mL}$. In another study, Hsin *et al.* (2008) discovered that AgNPs (< 250 μm) caused a significant increase in cell death in NIH3T3 fibroblast cell lines through an oxidative

stress-related mechanism. Their findings showed that nanosilver induced apoptosis through ROS and JNK in the mitochondria pathway.

Arora *et al.* (2009) in a bid to investigate cellular responses of dermal fibroblast cell and primary liver following exposure to spherical AgNPs of sizes 7 – 20 nm via a wound treatment gel. The authors observed that apoptosis was not induced but the AgNPs altered the antioxidant defense system in both cell types.

Immunological responses through investigation of cytokines is a proven tool as an indicator of toxic effects. Various studies have assessed toxicity via cytokines which are mediators of cellular immune responses. Responses such as increased macrophage inhibitory protein-2 (MIP2) and other cytokines have been reported in the macrophage cells of alveoli origin exposed to silver nanoparticles (Carlson *et al.*, 2008). Greulich *et al.* (2009) also reported alterations in the levels of cytokines in the stem cells after interaction with different concentrations of AgNPs. Similarly, responses of human epidermal keratinocytes and peripheral mononuclear cells to AgNPs included significant increase in cytokine productions, and inhibition of phytohaemagglutinin productions. Choi *et al.* (2011) assessed haemolytic properties of AgNPs on red blood cells. The authors discovered that its presence in the blood resulted in more red blood cell damage relative to the macro size form due to its size and surface properties.

In another cytotoxicity assessment, Mukherjee *et al.* (2012) exposed cervical cancer and human dermal cell lines to different concentrations of AgNPs. The AgNPs were reported to significantly increased ROS, depletion of glutathione and lipid peroxidation. The cell type was identified as an important factor that determined level of responses observed because different cell types exhibit different antioxidant levels.

The size of NPs has been described as a key factor that determines NPs toxicity. In a study involving the cytotoxicity of different sizes of AgNPs (10, 50 and 100 nm) in different cells (human cervical cancers, mouse osteoblastic and rat adrenal derived cells), were investigated (Kim *et al.*, 2012). The authors reported that toxicity increased significantly with decrease in size and increase in concentration in the cell lines used. Their report showed that the 10 nm sized particles had higher potential to induce cell death than the two other sizes.

Yue *et al.* (2015) assessed the toxicity of citrate capped -AgNPs using fish gill cell lines from rainbow trout (*Oncorhynchus mykiss*) in different aqueous solution of varying the ionic strength and chloride content. The authors observed correlation between rate of agglomeration of citrate capped-AgNPs and toxicity. The citrate capped AgNPs toxicity was attributed to the cytotoxic activity of the NPs and dissociated silver ion from the NPs in solution.

2.7.3.2 *In vivo* toxicity

Literature on the acute toxicity of AgNPs in freshwater vertebrate are limited. An investigation its acute toxicity (10 – 10 nanometer size) in zebrafish for 120 h led to size-dependent toxicity and morphological aberrations and LC₅₀ of 93, 126, 127 and 137 μM respectively. Similarly, in Zebrafish, LC₅₀ of 7.07 mg/L was reported following exposure to silver nanoparticles of size 26.6 nm for 48 h (Griffitt *et al.*, 2008). Kovriznych *et al.* (2013) conducted short term test on thirty-one types of NPs (including silver nanoparticles) using OECD203 and 210 on young and adult zebrafish (*Danio rerio*). They reported LC₅₀ values of 2.9 and 1.3 at 48 and 96 h respectively in the adult fish while acute toxic effects in the early life egg stage were 2.7 and 2.0 at 48 and 96 h respectively. Acute toxicity in the eggs led to malformations.

Contrastingly, Wu *et al.* (2010) reported a high toxicity of 100 % lethality at 2.0 mg/L in both adult and embryonic Japanese medaka exposed to 20 – 37 nm sized silver nanoparticles for 48 h. Authors reported an LC₅₀ value of 1.03 mgL⁻¹ was reported from the study. The high toxicity was considered to be an interspecies difference to the toxic effect of AgNPs. In a study, comparing the toxic effects of AgNPs and nanowires (AgNWs) on Japanese Medaka fish. an average 96-hour lethal concentrations (LC₅₀) of AgNPs and AgNWs were identified as 1.8 and 4.18 mg/L, respectively. The report indicated AgNPs were estimated to be 2.32 times more toxic than the AgNWs for *Oryzias latipes*. Although signs of AgNPs accumulation were visible in fish gills, no apparent accumulation was detected after AgNWs exposure (Sohn *et al.*, 2015)

Kim *et al.* (2013b) reported toxic effects of AgNPs (10 nm) in rat in accordance with OECD 423 test guideline. Authors discovered that doses up to 2000 mgkg⁻¹ bw were non lethal. This shows that oral LD50 is far way greater than 2000 mg/kg bw). Also, in another Maneewattanapinyo and colleagues (2011) carried out acute toxicity study

involving mice, the authors dosed mice with pure silver nanoparticles solution (10-20 nm,) and observed that the AgNPs did not cause any observable or lethal toxicity signs up to a 5000 mg/kg bw. Hence, they described its LD₅₀ AS far greater than 5000 mg/kg bw Report of Cha *et al.* (2008) showed that oral administration of 2.5 g of 13 nm AgNPs did not induce lethal toxicity but exposed mice exhibited some alterations in histological structures of the heart, liver intestine and spleen three days post treatment

An inhalation toxicity study also showed response of rats to twenty nanometers sized AgNPs in a chamber at 76 μgm^{-3} - 750 μgm^{-3} for a period of four h did not result in mortality nor were there tangible change in body weight. The authors reported that the LC₅₀ was greater than 750 μgm^{-3} which did not show any observable toxic effect (Sung *et al.*, 2011). However, the dose tested can be considered as relatively low and maybe responsible for the indeterminate result of the acute inhalation toxicity.

2.7.3.3 Sub-acute toxicity

A sub-acute effect (28-days) of 60 nm silver nanoparticles using the repeated dose oral administration in rats (at 30-1000 mgkg^{-1} bw) was reported by Jeong *et al.* (2010). They observed accumulation of AgNPs in the rats' intestines at the two highest concentrations. They also reported changes in the histopathological architecture of the rats' intestinal mucosa. Similarly, in another sub-acute test, repeated oral gavage of AgNPs (60 nm) was administered to mice (at same concentrations used by Jeong and colleagues) in carboxymethyl cellulose as the vehicle solution. The authors reported that the NPs administration did not affect the weight change of the mice. However, they observed that the cholesterol and ALP levels increased significantly in the blood stream of the mice at the two middle and highest concentrations and therefore suggested that it induced liver damage. In addition, the authors reported accumulation of Ag in the brain, lungs, kidney, stomach, liver, stomach testes and lungs of the mice. The females had higher silver concentration in the kidney compared to the male (Kim *et al.*, 2008).

In another study by Kim *et al.* (2010), rats were exposed orally to silver nanoparticles for 90 days. The authors reported 30 mgkg^{-1} bw/day as the NOAEL and 125 mgkg^{-1} bw/day as the LOAEL because it resulted in bile duct hyperplasia and changed the colouration of villi in the intestines

2.7.3.4 Dermal toxicity

Trop *et al.* (2006) reported that repeated use of Acticoat wound dressing kit which contained silver nanoparticles (15 nm) for a period of six (6) days (with a change of dressing on the 4th day) resulted into argyria and elevation in the liver enzymes concentration in the blood suggesting hepatotoxicity in the burns patient. This indicates absorption of the AgNPs through damaged skin. However, observed symptoms such as change in patient facial colour as well as increased liver enzyme concentration in the blood system were reversed after the patient discontinued the treatment.

In another human, Vlachou *et al.* (2007) examined 30 burn patients who had wound on about 12 % of their entire body surface following operation. The authors reported that the patients' use of acticoat, a material for wound dressings (which contained AgNPs for a maximum period of 28 days did not show any change in haematological or biochemical parameters. Also, the dermal treatment of mice with silver nanoparticles-based dressing (0.48 mg/12 m²) following thermal injury on a daily basis for a 25-day period minimized inflammation and other immunological changes associated with fibrinogen and hence faster healing of wounds without any adverse effect (Tian *et al.*, 2007).

2.7.3.5 Inhalation toxicity

Stebounova *et al.* (2011) evaluated the effect of inhalation of at 3.3 mg/m³ silver particles (5 nm) in rats, 4 h every day for two weeks (5 times /week). Authors observed minimal pulmonary inflammation or cytotoxicity in the exposed animals. Another study involving inhalation of AgNPs (approximately 15 nm) (1.2 x 10⁴ to 1.2 x 10⁶ particles/cm³ of AgNPs for 6 hr daily and 5 times weekly for a month) in rats which lasted for twenty-eight days did not induce visible toxicity related effects (Ji *et al.*, 2007).

Also, mice exposed to AgNPs (25 nm) through inhalation chamber at 1.91 x 10⁷ particles/cm³ to assess neurotoxicity in mice. The Silver nanoparticles administration did not cause any observable structural brain damage but some exposure response gene were expressed (Lee *et al.*,2010). Sung *et al.* (2008), exposed rats to AgNPs (20 nm) for 90 days to three different doses of 48.94 µgm⁻³, 133.19 µgm⁻³ and 514.78 µgm⁻³. The exposure did not affect organ and body weight, and haematological indices. In

another publication Sung *et al.* (2009) reported histopathological alterations in the pulmonary cells of rats exposed to same doses in the previous study (Sung *et al.*, 2008). They further observed bile-duct hyperplasia as well as perivascular infiltration in the liver. Song *et al.* (2012) also reported damage in rats at a dose of $100 \mu\text{g}/\text{m}^3$ following 12-week exposure. They detected lung functional and pathological changes. They authors reported $117 \mu\text{g}/\text{m}^3$ as the NOAEC for rats.

2.7.3.5 Reproductive and developmental toxicity

Silver nanoparticles has detrimental effects on both testes and ovaries. Accumulation of AgNPs was reported in testes of mice exposed to it (30 to $1000 \text{ mgkg}^{-1} \text{ bw/day}$) for twenty-eight days (Kim *et al.*, 2008). Mahabady (2012) in a bid to investigate the teratogenic effect of AgNPs in developing rat embryo, administered a dose of 0.4 and 0.8 mgkg^{-1} intraperitoneally to fraught rats at either day 8 or 9 d of gestation. The result showed significant reduction in weight and length of the foetuses removed on 20th day of gestation (of both doses of GD 8 as well as the highest dose of GD 9). In addition, the placenta width, volume and weights were lower in exposed animals compared the control experimental group.

Similarly, Austin *et al.* (2016) examined biodistribution properties of AgNPs (approximately fifty nanometers) in pregnant mice and developing foetuses after an intravenous injection on different days of gestation periods (doses of 35 and $66 \mu\text{g}$ silver per mouse on GD 7, 8 and 9). They study outcome revealed that AgNPs accumulated significantly in all organs/tissues, as well as the foetuses. Order of increasing accumulation observed were foetuses, maternal brain, visceral yolk, spleen and liver. in the visceral yolk, spleen and liver.

2.7.3.6 Genotoxicity of silver nanoparticles

Tables 2.2 and 2.3 highlights some effects of silver nanoparticles in different cell types, model plant and animals.

In vitro studies

In vitro investigations on effect of silver nanoparticle in mammalian cells have shown DNA damage. However, the mechanism of action is yet to be totally unraveled Reports

have shown reduction in DNA concentrations, and genetic damage in the human testis cell line (Asare *et al.*, 2012), genotoxicity induced via ROS and defect of DNA repair enzymes (Choi *et al.*, 2009), up regulation of proteins involved in DNA repair and direct inhibition

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Table 2.2. Some *in vitro* genotoxicity studies on silver nanoparticles

Coated/Uncoated	Size/shape	Genotoxicity test	Cell/ Cell line	Exposure concentration	Findings	Citations
AgNPs	5 -10nm	γ -H2AX assay;	Human hepatoma cell (HepG2)	1, 2 μ g/ml	Concentration-dependent increase in γ -H2AX phosphorylation which is an indication of damage to DNA Induction of genetic damage was Prevented by 10 mM NAC indicating Oxidative mechanism in AgNPs genotoxicity	Kim <i>et al.</i> , 2009
AgNPs	20nm	Comet assay	Human testicular embryonic carcinoma cell (NT2)	12.5 - 100 μ g mL ⁻¹ for 24h	Concentration-dependent induction of damage to DNA	Asare <i>et al.</i> , 2012
AgNPs	\approx 125nm	Comet assay	Human lymphocytes	25, 50, 100, 150 and 200 μ g mL ⁻¹	Genetic damage significantly induced at 25, 50 and 200 μ g/mL	Gosh <i>et al.</i> , 2012
AgNPs	40 – 50nm	Ames test MN and Cytokinesis-blocked micronucleus assay (CBMN) Comet assay	<i>S. typhimurium</i> strains (TA98, 100, 1535 and 1537) Chinese Hamster ovary cell (CHO-K1)	100, 200, 300 and 400 μ g per plate 0.01, 0.1, 1 and 10 mgmL ⁻¹ 0.01, 0.1, 1 and 10 mgmL ⁻¹	Mutagenicity was not detected. Significant increase in micronuclei induction in MN and CBMN assays Dose-dependent increase in DNA breakage	Kim <i>et al.</i> , 2013b

Coated/Uncoated	Size/Shape	Genotoxicity test	Cell/ Cell line	Exposure concentration	Findings	Citations
AgNPs	Spherical 40 – 60nm	Comet assay	Human peripheral lymphocytes	50, 100µg for 3h	Significant increase in DNA damage	Flowler <i>et al.</i> , 2012
AgNPs	5nm	Ames test Micronucleus test	<i>S. typhimurium</i> strains (TA102, 100, 98, 1535 and 1537) TK-6 cell	0.15 – 76.8µg/plate for 4h 10, 15, 20, 25, , 30 µg/ml	No increase in mutagenic cells Significant induction of micronucleus	Li <i>et al.</i> ,2012
AgNPs	10, 20, 50 and 100nm	Ames test MN Test Comet assay	<i>S. typhimurium</i> strains (TA100, TA98, TA102) and <i>E. coli</i> (WP2Pkm101 and uvrA/pKM101) Jurkat E 61 THP – 1	10, 20, 40 and 100µg/L for 24 h	Negative mutagenicity in the bacteria cells Size and concentration-dependent increase in MN induction in both mammalian cells. DNA damage was induced at 10nm and 20nm AgNPs	Butler <i>et al.</i> ,2015

Coated/Unc oated	Size	Genotoxicity test	Cell/ Cell line	Exposure concentration	Findings	Citations
AgNPs (either PVP or Citrate coating)	20, 30, 100nm	Ames test Mouse Lymphoma Assay MN	<i>S. typhimurium</i> strains (TA98, TA100) L5178Y cells TK -6 human lymphoblastoid cell lines Cells	6.3 - 100 µg/plate for 4 h 1 – 60 µg/ml for 4h 2.5 - 400µg/ml for 4h	Negative mutagenicity Size-dependent genotoxic effect in the MLA and increased MN frequency in both L5178Y cells and TK – 6 cells;	Guo <i>et al.</i> , 2016

Table 2.3. Some *in vivo* genotoxicity studies on silver nanoparticles

Coated/Uncoated	Size/Shape	Genotoxicity test(s)	Animal /Plant Model	Exposure route /concentration	Findings	Citations
AgNPs	<100 nm	Chromosome aberration	<i>Allium cepa</i>	25, 20, 75, and 100 ppm	Induction of different chromosomal aberrations and significant dose dependent decrease in mitotic index	Kumari <i>et al.</i> , 2009
AgNPs dispersed in anionic surfactant (AOT)	9±6 nm	Sperm head abnormality test Neutral Comet assay	Male and female BALB/C mice	Single intraperitoneal dose at 5; 3.3; 2.5; 1.6; 1.0; 0.7; 0.5; and 0.05 · (10 ⁻³ g·ionL ⁻¹) for 30 days period	Both AgNPs solution and its dispersant(AOT) Induced significant frequency of abnormal sperm head and primary DNA damage. Non - significant difference in genotoxic damage in AgNPs solution and AOT	Ordzhonikidze <i>et al.</i> ,2009
AgNPs	≈125 nm	Chromosome aberration (CA); Comet assay Comet assay	Male Swiss Albino mice; <i>Allium cepa</i> and <i>Nicotiana tabacum</i>	Single IP injection at 10, - 80 mg/kg 18 h prior to sacrifice 25, 50 and 75 µg mL ⁻¹ for 24 h	The genotoxic response was due to the dispersant Induction of CA at all concentration and increased DNA damage at 10 and 20mg/kg in bone marrow cells significant Induction of genetic damage in both root (at 25 and 50 µg mL ⁻¹) and shoot (25 µg mL ⁻¹ of <i>A. cepa</i> Significant DNA damage in roots (50 and 75 µg/mL) and shoots	Gosh <i>et al.</i> , 2012

Coated/Uncoated	Size/Shape	Genotoxicity test(s)	Animal /Plant Model	Exposure route /concentration	Findings	Citations
AgNPs	10 nm	Comet Assay; Chromosome aberration assay; micronucleus test	Male Sprague – Dawley rats	Oral exposure at 5, 25, 50 and 100mg/kg for 5 consecutive days	Significant increase in DNA damage (50 and 100mg/kg), structural chromosome aberrations and micronuclei frequencies (50 and 100mg/kg) and decreased rate of cell division compared to controls (at 25, 50 and 100mg/kg)	Patlolla <i>et al.</i> , 2015b
AgNPs	18nm	Bone marrow micronucleus test	Female and male Sprague Dawley rats	Exposure via inhalation route at 0.7×10^6 , 1.4×10^6 and 2.9×10^6 particles/cm ³ for 6 hr/day for a 90-day period	No statistically significant increase in micronucleated cells and cytotoxicity index	Kim <i>et al.</i> , 2011
AgNPs	63±41nm	Chromosome aberration Micronucleus test	<i>Vicia faba</i>	12.5, 25, 50 and 100mg/L	Significant concentration-dependent increase in chromosomal aberrations and micronucleus inductions	Patlolla <i>et al.</i> , 2012
AgNPs	20 nm 200 nm	Sperm morphology test Comet assay	Male Wistar rats	Intravenous injection of 5 and 10 mg/kg for 24 h, 7, and 28 days	No significant change in frequency of observed sperm morphology but it induced dose and time dependent DNA damage in sperm cells Smaller size NPs exerted more damaging effects.	Gromadzka-Ostrowska <i>et al.</i> , 2012

Coated/Uncoated	Size/Shape	Genotoxicity test(s)	Animal /Plant Model	Exposure route /concentration	Findings	Citations
AgNPs	4.23 - 46.95 nm	Comet assay	Male, Sprague Dawley rats	Exposure via inhalation chamber at 0.6×10^6 , 1.4×10^6 and 2.5×10^6 particles/cm ³ for 6 hr/day for 12 weeks	Significant DNA damage at highest dose compared to control	Cho <i>et al.</i> , 2013
AgNPs (citrate coated)	21 – 41nm	Sperm morphology assay	Male mice	Intraperitoneal exposure at 100, 500 and 1000mg/kg for 28 days	Significant dose – dependent induction of sperm morphology	Attia, 2014
AgNPs	<100nm	Micronucleus test (peripheral blood)	Female ICR mice	Intraperitoneal injection at 1 and 3mg/mouse for 72 h	Increased levels of 8-OH-dG in bone marrow and DNA liver with peak values at 24 h for CuONPs group but not in the other NP groups	Song <i>et al.</i> , 2012
AgNPs and	20 ± 5 nm	Comet assay 8-OH-dG Micronucleus assay	Adult male wistar rats	Single intravenous injection of 5 and 10mg/kg AgNPs (20nm) 5mg/kg (AgNPs, 200 nm)	Tail comet and percentage DNA were not significantly different but slightly enhanced in exposed compared to control Significant increase MN frequency at 10mg/kg in the AgNPs group after 1 and 4 weeks	Dobrzynska <i>et al.</i> , 2014

Coated/Uncoated	Size/Shape	Genotoxicity test(s)	Animal Model	/Plant	Exposure /concentration	route	Findings	Citations
AgNPs	< 100 nm	Comet assay	Mussels (Mytilus galloprovincialis)		One dose level exposure of 10µg/l for 3, 7 and 15 days		Significant increase in DNA damage following 7 days exposure.	Gomes <i>et al.</i> , 2013

of DNA replication by silver nanoparticles its adherence to the genetic material have all been documented (Yang *et al.*, 2009).

Park *et al.* (2011) reported that silver nanoparticles (20, 80 and 113 nm) inhibited the differentiation of murine embryonic stem cells in a concentration dependent manner. The authors observed that smallest nanoparticle size had the higher inhibition effect on the cell differentiation. However, the ionic form proved more cytotoxic as it showed highest level of cell inhibition. In addition, smallest size of the tested nanoparticles induced transferrable damage in murine fibroblast cell. This further buttress the importance of size in cell toxicity.

Li *et al.* (2012) investigated mutagenicity and genetic damaging effect of AgNPs in five Salmonella strains and human lymphoblastoid TK6 cells using the Ames test (OECD TG 471) and an *in vitro* MN assay (OECD TG 487) respectively. Authors reported negative results for the mutagenicity tests in five Salmonella strains (at 2.4 to 38.4 μg per plate). However, AgNPs induced micronuclei formation in the human lymphoblastoid cells in a concentration-dependent manner at 10 – 30 $\mu\text{g mL}^{-1}$ but was a weak genotoxic effect. The authors attributed the observed negative result in the Ames test to the barrier created by the bacterial cell wall and the lack of sensitivity of the bacteria strains tested. They therefore regraded the *in vitro* MN test as a more sensitive for genotoxicity evaluations of nanoparticles in comparison with the Ames test.

In vivo studies

Two studies (oral and inhalation) carried out by Kim *et al.* (2008 and 2011) assessed genotoxicity of silver nanoparticles (60 nm) in rats after 28 days oral exposure using the rat bone marrow micronucleus assay. The oral exposure of rats to 30, 300, and 1000 mg kg^{-1} bw/day to the nanoparticles did not show no significant effect on micronuclei induction in both erythrocyte and bone marrow cells. The inhalation experiment also, did induce micronuclei damage in the following a 90 days exposure.

Song *et al.* (2012) reported an increase in the induction of MN in peripheral reticulocytes of mice exposed to single dose of AgNPs (3mg/kg). Dobrzynska *et al.*

(2014) reported that single intravenous exposure of rats to TiO₂ and AgNPs significantly induced micronuclei formation at twenty for h post- exposure, and at 1 and 4 -week exposure in the AgNPs group. In contrast, comet assay result of the same experiment was negative in the bone marrow leukocytes. Furthermore, Patlolla *et al.* (2015b) reported significant induction of MN and DNA damage (comet assay) as well as structural chromosome aberrations in adult Sprague Dawley rats that were orally administered AgNPs (10nm) for 5 consecutive days.

Tavares *et al.* (2012) carried out DNA damage assessment of silver particles (average 19.7 nm) in laboratory mice and human blood cell using the comet assay. The result showed that the silver nanoparticles induced DNA damage at all tested concentrations (10-50 µg mL⁻¹) at the initial post treatment assessment (1hr) which decreased with increasing time. They suggested that the DNA damage repair mechanism might be responsible for the observed response. However, the mice study showed negative results. The authors suggested that it may have been due to activation of the antioxidant molecules/enzyme system that prevented the damage.

In another study, the authors investigated the genotoxicity of silver nanoparticles in Sprague- Dawley rats using the comet, chromosome aberration and bone marrow micronucleus assay. AgNPs administered orally at 5-100 mg/kg for five days significantly induced ROS, structural chromosomal aberration, increase in the frequency of micronucleated cell (Patlolla *et al.*, 2015b).

In vivo plant systems

Genotoxicity studies in higher plants have also shown potential genotoxicity of AgNPs. Kumari and colleagues (2009) and Patlolla *et al.* (2012) used *Allium cepa* and *Vicia faba* models respectively to reveal genotoxic damage of AgNPs (< 100 nm and 63 nm) in roots of the plants. After 4 h, AgNPs significantly inhibited rate of cell division and increased the number of aberrant chromosomes in both experiments. These studies, however, did not take into consideration exposure of the meristematic root tips over complete mitotic cycles which last for about 16 – 24h in *A. cepa* (Fiskesjo, 1985) and 15- 18 h in *Vicia faba* (Bennett *et al.*, 1972). However, Pesnya (2013) exposed *A. cepa* root tip to varying concentration of chitosan-coated AgNPs (1, - 50 mg L⁻¹) for 96 h and observed that the chitosan-coated AgNPs (size: 10–30 nm;

organic coat: 2–5 nm).did not induce cytogenotoxic effects w below 5 mg L⁻¹. The authors however observed mitotic and chromosomal abnormalities at 50 mg L⁻¹. In contrast to previous reports, there was a significant increase in the mitotic index at 5 and 50 mg L⁻¹. Also, another study showed that biogenically synthesised AgNPs from screw pine induced apoptosis, genotoxicity and ROS in *Allium cepa* (Panda *et al.*, 2011)

Aquatic vertebrates

Recently, Khan *et al.* (2017a) investigated the genotoxicity of AgNPs (17.78±12.12 nm) (which were synthesized in the laboratory from silver nitrate reduction using formaldehyde) in peripheral blood of *Labeo rohita*. The authors exposed *L. rohita* to 10–55 mg /L concentrations through in laboratory tanks for 14 and 28 days. AgNPs significantly induced micronucleus and other abnormal formations of the nucleus in the fish erythrocytes and concomitantly increased MDA and decreased GST. Authors concluded that AgNPs induced both oxidative stress and DNA damage. Similarly, in another study, authors investigated the genotoxic effect of AgNPs on juvenile fish *Piaractus mesopotamicus* (“pacú”) using the single cell gel electrophoresis assay and assessment of oxidative damage, metal burden, and antioxidant enzymes. Following 24 h short-term exposure to AgNPs (0 - 25µg/L). AgNPs significantly induced an increase in comet tail, MDA and metal burden in different organs like the gills, brain and liver in comparison with the control experiment (Bacchetta *et al.*, 2017).

2.7.3.7 Systemic toxicity of silver nanoparticles

Mammalian rodents

The biocidal property of AgNPs has been associated release of silver ion following its dissolution (Xiu *et al.*, 2012). Its administration in the mice resulted in AgNPs accumulation in various organs indicating its potential in the biological system. Kim *et al.* (2008) reported a presence of silver in blood and body organs/ tissues from the circulatory, excretory, respiratory, digestive, reproductive and excretory system, and nervous system as a sign of systemic toxicity following a sub-acute oral exposure to silver nanoparticles. Similarly, oral exposure of rat to AgNPs (10-18 nm) coated with polyvinyl pyrrolidone also accumulated in same organs (Loeschner *et al.*, 2011).

However, they also detected that more than 50% of the AgNPs intake were excreted via faeces.

Findings of Lubic, (2012) in male rats fed either with silver nanoparticle coated with polymer, uncoated nanoparticles or a silver nitrate solution for 28 days showed that silver ion were much more readily absorbed compared to the NPs. The author reported ten times higher levels of silver in the spleen and livers tissues in rats administered AgNO₃ than those administered NPs. They also discovered that silver transforms between ions and the nanoparticulate forms in the animal tissues. Likewise, sub - chronic experimental exposure of rats via inhalation of AgNPs aerosol (18 -19 nm; 49- 515 µg/ m³) also resulted into accumulation in similar organs as those of Kim *et al.* (2008). They reported that silver was enormously cleared from the lungs by the seventh day after exposure but entered the systemic pathways. The initial silver concentration in the lungs was reduced to about 4%. Furthermore, they detected silver in lower concentration in the kidney, heart, liver spleen and brain but found higher silver concentrations in the posterior portion of the nasal cavities and lymph nodes associated with the lung (Takenaka *et al.*, 2001). In another study, in mice, radio labelled AgNPs were monitored and found in liver and spleen barely 24 h post exposure (Lankveld *et al.*, 2010). Similar observation occurred following five-day intravenous administration of 20 – 110 nm sized silver nanoparticles to rats also resulted in the accumulations of silver nanoparticles in the spleen, lungs and liver (Lankveld *et al.*, 2010)

An investigation on the effect of NPs size on toxicity was done by Park and colleagues (2010a). They administered silver nanoparticles (22, 42, 71 and 323 nm) to mice at a concentration of 1 mgkg⁻¹ bw/day. The authors observed that smaller sized silver (22, 42 and 71 nm) were distributed via the blood stream and accumulated in vital organs of the digestive, respiratory, excretory and reproductive system; and stimulated inflammatory responses. They further reported that larger sized particles (323 nm) because of its big size was not detected in tissues of the exposed animals. However, the AgNPs did not alter the body and organ weight across the treated group. The authors went a step further to investigate the sub-acute exposure of 42 nm of same particles to mice 0.25 - 1 milligram per kg body weight. Their result showed that significant elevation in the activities of the liver and kidney function enzyme

indicators at the highest dose. This shows that AgNPs is capable of causing hepatic injury. In addition, pro-inflammatory cytokines level increased and histopathological alterations occurred in the kidney at the same concentrations.

El - mahdy *et al.* (2014) exposed albino rats intraperitoneally to 8.7 nm size AgNPs which presented with histopathological lesions and oxidative damage in form of MDA. Levels of GSH was also significantly altered. AgNPs also induced chromosome aberrations of different types in the rats' bone marrow. Adeleye *et al.* (2014) reported that AgNPs – exposed mice to oral doses of 100, 1000 or 5000 mgkg⁻¹ for 7 or 14 days, and 5000 mgkg⁻¹ for 21 days, significantly induced lipid peroxidation and altered antioxidant enzyme activities. These shows potentials of AgNPs to cause oxidative stress. In another study, the biochemical effects of silver nanoparticles (< 100 nm) was also explored in Wistar rats at 100, 1000, and 5000 mgkg⁻¹ daily administration for 7, 14, and 21 days. The authors showed that AgNPs did not produce significant loss of appetite and body weight but caused altered the activities of serum and tissue AST, ALT, and ALP (Adeyemi and Adewumi, 2014).

Patlolla *et al.* (2015a) also reported that 5 - day acute administration of AgNPs via oral gavage to rats (5, 25, 50 and 100 mg kg⁻¹ AgNPs) elicited oxidative stress, increase in comet tail migrations, concentrations of the hepatic injury biomarkers (AST, ALT, ALP), histological changes in the liver tissue in the exposed rats. They concluded that short-term exposure to Ag-NP may induce toxic effects.

In a study assessing neurotoxicity of silver nanoparticles, Rats exposed via inhalation of AgNPs (15 nm) for 6 h revealed that the nanoparticles crossed the all barrier and was detected in the main organ of the nervous system (Takenaka *et al.*, 2001). Also, the injection of AgNPs into the rats' sub – cutaneous layer (< 100nm; 62.8 mgkg⁻¹ bw) resulted in accumulation of silver in the brain alongside other organs. Assessment of silver content in the brain and the other organs showed significantly higher concentrations than the unexposed between 8-24 wks after exposure. Also, the accumulation was accompanied with swelling of the astrocytes and degeneration of the neuron in exposed rats between three weeks after administration (Tang *et al.*, 2009). Exposure to silver nanoparticles (50 – 60 nm) via different exposure routes were explored by Sharma *et al.* (2009). Authors reported that intraperitoneal (50 mg/kg bw),

intracerebroventricular (20 µg) and intravenous (30 mg/kg bw) administration of the silver nanoparticles resulted in compromise of the blood brain barrier. They further reported that the neurotoxic effects were severe in mice compared to rats.

Aquatic vertebrates

Reports of Wu *et al.* (2010) has shown that silver nanoparticles can cause developmental toxicity in embryo of *Oryzias latipes* in form of edema, malformations in the spines, eye, heart and brain. Wu and Zhou (2013) also reported the bioaccumulation of AgNPs in gills, liver and intestines of Japanese medaka fish exposed for 14 days. Other observed effects include a concentration dependent decrease in lactate dehydrogenase, alteration of antioxidant molecules and enzymes activities in the hepatocytes and gill cells; and hepatic histological lesions among others. This indicates potential toxicity of AgNPs and oxidative stress. However, Park *et al.* (2013) reported that citrate capping in AgNPs conferred stability in AgNPs for 7 days in diluted fish water and also inhibited significant adverse effect in zebrafish. Recently, Rajkumar and other scientists (2016) assessed the impact of oral administration of AgNPs (50 – 100 nm) on haematological, biochemical and histopathological properties in *Labeo rohita*. The oral administration AgNPs (25 – 1000 mg/kg) showed an LC50 of 100 mg/kg after 7 days. AgNPs induced dose-dependent increase in bioaccumulation of silver in gill, liver and muscle as well as haematological and histological alterations.

2.8 Copper(II) oxide nanoparticles

Copper(II) oxide nanoparticle is the least complex member of Copper compounds. It is blackish in colour. As a compound, it comprises two elements: copper and oxygen (Figure 2.3) and has attracted attention as a semiconductor with monoclinic structure. Its crystal structure with a narrow band gap confers on it photovoltaic properties and photoconductive properties. Also CuONPs is useful in improvement of fluid viscosity and enhancement of thermal conductivity which makes them energy-saving materials in energy conversion (Chang *et al.*, 2012). CuONPs show superior temperature, superconductivity and catalytic activity than that of copper(II) oxide (CuO) powder. It possesses composition of Cu: 79.87% and O2: 20.10% and a Molar mass: 79.55g/mol. Melting point of CuONPs is 12010C (21940F). other

properties include: boiling point: 2000C (36320F), Refractive index: 2.63, Bandgap: 1.2eV, thermochemistry: Standard molar entropy (S₀₂₉₈)- 43J-1mol/K , density: 6.315 g/cm². . It is not soluble in water but soluble in ammonium chloride, potassium cyanide (Singh *et al.*, 2016).

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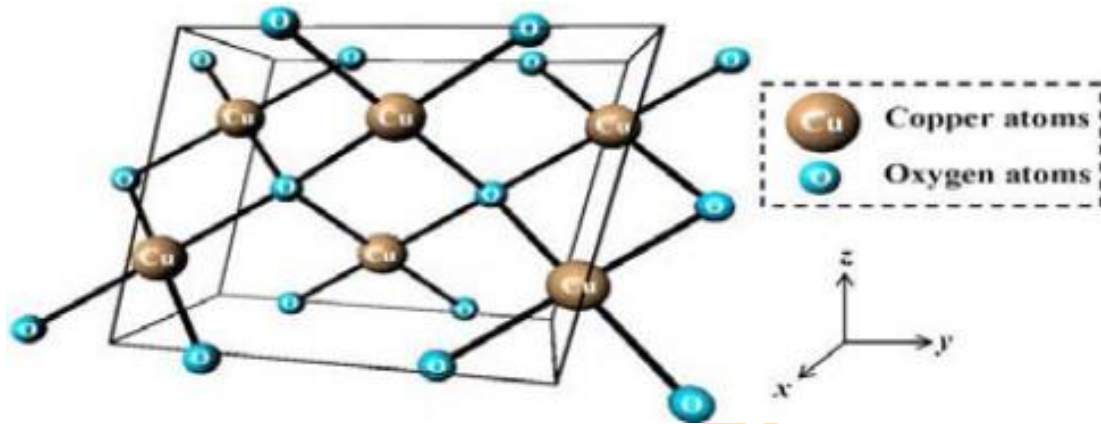


Figure 2.5. Schematic representation of monoclinic copper(II) oxide unit cell
Source: Singh *et al.* (2016)

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2.8.1 Applications of copper(II) oxide nanoparticles

CuONPs are been utilized in the numerous applications/products like batteries, gas sensors, catalysis, superconductors, solar panels, field emission emitters. In addition, it's a replacement for noble metal catalyst in industrial catalysis because it reduces cost of production and efficiency of catalysis. Also, due to its thermophysical properties, they are useful in heat transfer fluids; as additives in lubricants, plastics and coating of metals (Chang *et al.*, 2005; 2012). Furthermore, they possess biocidal property which makes them useful as antimicrobial agents in a number of products. Reports on its antimicrobial properties have been reported in *Escherichia coli* strains (Pan *et al.* 2010). As a result of the aforementioned properties, they are found in face masks, wound dressings and socks (Burkow *et al.*, 2009;2010). They are also used in wood preservation because they possess antimicrobial properties (Gabbay *et al.*, 2006).

2.8.2 Synthesis of CuONPs

Copper(II) oxide nanoparticles are synthesized by numerous techniques some of which are pulsed laser ablation, vacuum vapor deposition, pulsed wire discharge and mechanical milling can be classified as physical techniques. Techniques such as microemulsion techniques, sonochemical, electrochemical, microwave assisted and hydrothermal methods are chemical approaches for the synthesis of this nanoparticles. Biological or biosynthesis techniques are also considered as chemical methods (Davaranah *et al.*, 2015).

2.8.3 Toxicity and genotoxicity of CuO nanoparticles

Cu is an important element needed in the biological system but when its level is high, it may lead to serious health effects. which makes it great public health concern (Chang *et al.*, 2012). Information on toxicity and genotoxicity of CuONPs is insufficient and most of the available literatures are *in vitro* toxicity studies. Tables 2.4 and 2.5 shows reports of genotoxicity studies on CuONPs carried out in the living tissues and in cell media.

Karlsson *et al.* (2008) compared the cytotoxicity of different metal- oxide NPs (CuO, TiO₂, ZnO, CuZnFe₂O₄, Fe₃O₄, and Fe₂O₃) using trypan blue for cell viability and reported that CuONPs was the most toxic. Karlsson and colleagues reported that CuONPs was cytotoxic and genotoxic in the A549 cell line which is of epithelial origin in human lungs. In another study, authors implicated ROS generation in the genetic damage (Wang *et al.*, 2012a). Similarly, mutagenicity evaluation of Al₂O₃, Co₃O₄, TiO₂, ZnO and CuO nanoparticles in *S. typhimurium* TA97a, TA100,

and *E. coli* WP2 trp Uvra revealed that only CuO exhibited mutagenicity though it was a weak one to *S. typhimurium* and significant dose-dependent inhibition to *E. coli* at the tested concentrations; the other four metal oxide nanoparticles did not induce mutagenicity in the three bacteria strains (Pan *et al.*, 2010).

CuONPs was reported to have adverse effects on bacteria through copper ions that dissociated from CuONPs. The copper ions elicited ROS and damage to the genetic material in bacteria (Bondarenko *et al.*, 2012). Perreault and colleagues (2012), reported genotoxic effects of CuONPs (30-40 nm; 6.25 - 400 mg/L) in Neuro2A cells in form of micronuclei formation in concentration-dependent manner. The authors also observed lipid peroxidation at tested concentrations. They resolved that the micronucleus test is sensitive in detection of CuONPs genotoxicity. Zhang *et al.* (2016) investigated the genotoxicity of CuONPs with different surface chemistry (CuO-Core, CuO-COOH, CuO-NH₂ and CuO-PEG) between 1 and 10 µg/mL⁻¹ in mesenchymal stem cells (MSCs) from rat's bone marrow for 3, 24 and 72. The authors reported that CuO-PEG only induced a dose – dependent increase in genotoxicity at 1 and 2 µg/mL. They concluded that genotoxicity of CuONPs tested was based on the dose and surface chemistry of the nanomaterial.

Akhtar *et al.* (2016) tested CuONPs cytogenotoxicity in A549 cell lines at 5-15 µg/mL. The authors reported a significant increase in level of genetic damage in form of tail comets and micronucleus induction. They concluded that the observed DNA damage and micronuclei induction occurred as a result of excess production of ROS, lipid peroxidation with concomitant depletion of glutathione.

Table 2.4. Some in vitro genotoxicity studies copper(II) oxide nanoparticles

Coated/un coated	Size/Shape	Genotoxicity test	Cell/ Cell line	Exposure concentration	Findings	Citations
CuONPs	52.51±10.23nm	Western blot analysis of expressed Hsp 70 and DNA damage markers (p53, Rad 51, MSH2 proteins)	Human lung epithelial (A549) cells	10 - 50 µg/mL ⁻¹	DNA damage induced via upregulation of Hsp 70 and p53 proteins	Ahamed <i>et al.</i> , 2010
CuONPs	20 to 200 nm.	Comet assay	rainbow trout (O. mykiss) red blood cells;	7.5 µg/ml for 1 h	significantly increased in percentage of tail DNA (DNA damage)	Isani <i>et al.</i> , 2013
CuONPs	Spherical; 22nm	qPCR and Western blot analysis of expressed DNA damage markers (p53, Bax, bcl2 and caspase 3)	Human hepatocarcinoma cell (HepG2)	2, 5, 10, 25 and 50 µg/mL	Up-regulation of p53 gene and apoptotic gene caspase 3, increase bax/bcl2 ratio	Siddiqui <i>et al.</i> , 2013

Coated/un coated	Size/Shape	Genotoxicity test	Cell/ Cell line	Exposure concentration	Findings	Citations
	30 – 40 nm	Comet assay; Micronucleus assay	Mouse neuroblastoma cell line (N2A)	6.25, 12.5, 50, 100, 200, 400 mg/L	DNA damage significantly increased at 6.25 - 50 mg/L Significant increase in micronuclei frequency at 12.5 – 100 mg/L	Perreault <i>et al.</i> , 2012
	20- 40nm	Real-time polymerase chain reaction and Comet assay	Human lung epithelial (A549) cells	15mg/L for up to	Increased expression of <i>p38</i> and <i>p53</i> gene expression Time-dependent increase in DNA damage	Wang <i>et al.</i> , 2012b
	50nm	Comet assay	human skin epidermal (HaCaT) cells	5, 10, 20 μ g ml ⁻¹ for 24 and 48 h	Significantly increased DNA damage	Alarifi <i>et al.</i> , 2013
	23 nm	Comet assay; Micronucleus assay	Human lung epithelial (A549) cells	5, 10 and 15 μ g mL ⁻¹	A concentration-dependent increase in DNA damage (tail comets) and micronuclei frequency	Akhtar <i>et al.</i> , 2016
CuONPs	15 -20nm and 70nm	Comet assay	Mouse macrophage cell (J774)	5.5% w/v 5.0%w/v	CuONPs but not TiO ₂ NPs induced significant DNA damage	Triboulet <i>et al.</i> , 2015
TiO ₂	25nm					

Table 2.5. Some *in vivo* genotoxicity studies in copper(II) oxide nanoparticles

Coated/ uncoated	Size/Shape	Genotoxicity test(s)	Animal /Plant Model	Exposure route /concentration	Findings	Citations
CuONPs	27.2 – 95.3nm	Micronucleus test (peripheral blood) 8-OH-dG	Female ICR mice	Intraperitoneal injection at 1 and 3mg/mouse for 72 h	Significant induction of micronuclei in reticulocytes (MNRETs) with peak value after 48 h post administration Positive increases in 8- OH-dG in urine in all NP tested at 3mg with 1.6 fold increase in the CuONPs group Increased levels of 8-OH- dG in bone marrow and DNA liver with peak values at 24 h for CuONPs group	Song <i>et al.</i> , 2012
CuONPs	< 50 nm (29.84 ± 15.28 nm)	Comet assay Wing-spot test	Fruit fly (<i>Drosophila melanogaster</i>)	0.24, 0.48 and 0.95 mg/mL	Significant increase in DNA damage and frequency of total mutant spots	Carmona <i>et al.</i> , 2015.

Coated/ uncoated	Size/Shape	Genotoxicity test(s)	Animal Model	/Plant	Exposure /concentration	route	Findings	Citations
CuONPs	< 50 nm.	Comet assay	Mussels (<i>Mytilus galloprovincialis</i>)		One dose level exposure of 10µg/l for 3, 7 and 15 days		Significant increase in DNA damage following 7 days exposure.	Gomes <i>et al.</i> , 2013
CuONPs	<50 nm	RAPD	Buck (<i>Fagopyrum esculentum</i>)	wheat	0, 50, 500, 2,000, and 4000 mg L ⁻¹ .		RAPD conducted at 2000 and 4000 revealed significantly lower averaged Nei genetic index (NGI) values for ZnONPs and CuONPs	Lee <i>et al.</i> , 2013
CuONPs	20 to 200 nm	Comet assay	Rainbow trout (<i>O. mykiss</i>)		Intraperitoneal injection of the equivalent mass of Cu = 1 µg g ⁻¹ body weight for 48h		No significant DNA damage was not observed compared to control	Isani <i>et al.</i> , 2013
CuONPs	< 50 nm	Micronucleus test	<i>Oreochromis niloticus</i>		7.5 mg/L and 150mg/L for 30 days		Significant induction of MN in comparison to control and bulk CuO	Abdel-Khalek, 2016

Aruoja *et al.* (2009) reported the toxicity of CuONPs on the algae *Pseudokirchneriella subcapitata* in comparison with its bulk form. At low concentrations, it was observed that CuONPs ($EC_{50} = 0.71 \text{ mgL}^{-1}$) were more soluble and toxic compared with the bulk form ($EC_{50} = 11.55 \text{ mgL}^{-1}$). The authors highlighted that the toxicity of both the bulk and nanosized CuO were dependent on Cu^{2+} to a large extent. Griffitt *et al.* (2007) in their study on effect of CuNPs reported similar toxicity in the fish *Danio rerio*. The authors also showed that the Cu^{2+} was very toxic to fish. Furthermore, reports have shown that CuNPs suspensions can cause damage in gill system.

The potential genotoxic effect of CuONPs were tested in the bivalve, Mussel (*Mytilus galloprovincialis*) for two weeks at $10 \mu\text{g mL}^{-1}$ (Gomes *et al.*, 2013). The authors observed that CuONPs induced DNA damage in a time-dependent pattern. They further noted that the ions released from the CuO NPs had a relatively higher genotoxic effect in the test organism than the NPs. It was concluded that the damage occurred generation of ROS which led to oxidative damage. Abdel - Khaleek (2016) exposed tilapia to 7.5 and 15 mg/L CuO (<50 nm) for 30 days and discovered that it induced MN in the fishes' peripheral blood erythrocyte and other nuclear anomalies.

Ates *et al.* (2014) assessed the potential acute toxic effect of copper(II) oxide nanoparticle (40 nm) in salt water using the fish sheepshead minnow as a model. Ates and colleague observed that cause behavioural changes in fish but was not lethal at tested concentrations (5 and 50 mg/L) and salinity strengths (1.5 and 3.0 %). Their findings showed that toxicity of copper(II) oxide(ii) nanoparticle in saline environment decreases as salinity increases. However, Cu accumulations were detected in the fish's intestine, gills and liver.

Shi *et al.* (2011) showed that CuONPs negatively affected duckweed by decreasing its chlorophyll contents. However, the authors reported that CuONPs was highly toxic than ionic form basically because of its higher dissociation rate.

Yokohira and colleagues (2009) studied pulmonary effects of CuONPs on rats' lungs via intratracheal instillation of CuONPs. The authors' result showed that CuONPs induced both severe acute and chronic inflammation in the rats' lung at high and low doses respectively. In a similar study, the exposure of cells to CuONPs disrupted the antioxidant defences system (CAT, Reduced Glutathione), generated ROS and

blocked the cellular antioxidant defence. Also, waterborne Cu induced apoptosis and necrosis as well as invasion of intercellular spaces by a huge number of white blood cells (Li *et al.*, 1998).

In addition, the genotoxicity of CuONPs was assessed in *Drosophila melanogaster*. The authors assessed alteration in the DNA and the antioxidant enzyme activities. The authors carried out the investigation using the wing spot test, comet assay, and lipid peroxidation evaluation. Their findings revealed genotoxic effects of CuONPs in the haemocytes, mutations in form of mutant spots and membrane oxidative damage (Carmona *et al.*, 2015).

2.9 Mechanism of nanoparticle-induced genotoxicity

The mechanisms of NPs' genotoxicity are yet to be fully understood. Genotoxicity can be categorised into primary and secondary genotoxicity (Magdolenova *et al.*, 2014). Primary genotoxicity occurs from interaction of between nanoparticles and DNA while the indirect genotoxicity refers to genotoxic damage arising from ROS produced by the NPs, or dissociated ions from NPs. A secondary genotoxic effect may arise via oxidative damage on the genetic material by ROS from the activated phagocytes during NP- induced inflammatory reactions (Magdolenova *et al.*, 2014).

As a result of its tiny size, NPs can cross nuclear membranes upon entry into the cell. This can occur by diffusion from cytoplasm across membrane to nucleus, thereby having the opportunity to interact directly with DNA. NPs (larger than nuclear pore size) can also interact with the genetic materials during process of mitosis following dissolution of the nuclear membranes or deform the nucleus (Di Virgilio *et al.*, 2010).

2.9.1 Direct primary genotoxicity

This might have occurred during DNA replication. Hence, NPs could disturb DNA replication when they come in direct contact with the DNA during mitosis. Interaction could occur at interphase stage between the NPs and DNA molecule thereby influencing replication and transcription to RNA (Figure 2.6). Report of An and colleagues (2010) reported that carbon NPs bound to DNA in *Escherichia coli* becoming an integral part of it. Interaction with the chromosome structure can also occur during mitosis causing chromosome break (clastogenic effect) or hindrance to

proteins or spindle fibre leading to the lagging of chromosome (aneugenic effect) (Magdolenova *et al.*,2014).

Moreover, genotoxicity could also be induced via primary indirect contact with the DNA (Figure 2.7). This can occur via interaction between NPs and the proteins that take part in the transcription, replication or repair processes. For instance, a modelling study on the interference of fullerene with the DNA topoisomerase elucidated that the binding of C60 fullerene to human DNA topoisomerase II alpha in the ATP binding domain, could result into inhibition of the activity of enzyme. The DNA topoisomerase II is an active protein that takes part in modification of DNA topology (Baweja *et al.*, 2011). Similarly, Gupta *et al.* (2011) published an *in-silico* report highlighting C60 fullerene as capable of interacting with important proteins that mediates the DNA mismatch repair. In addition, production of ROS by NP is also capable of inactivating proteins through structural modification (Jugan *et al.* 2012).

Another means of direct primary genotoxicity is the damage induced by NPs on mitotic spindle fibres and other associated proteins. This leads to aneugenic effects. *In vitro* experimental studies has shown that NPs can disrupt the mitotic process of division (Huang *et al.*,2009). The authors observed abnormalities in chromosome positioning and movement during mitotic phases, multipolar spindle formation as consequences of exposure to TiO₂NPs for a long duration. Another study by Gonzalez *et al.* (2010) also evaluated the aneugenic effect of NP on spindle fibres in human epithelial cells A549. Their findings confirmed that interaction with tubulin polymerisation could lead to aneugenicity.

The cell has different cycle checkpoints which control and monitors the integrity of DNA being passed from one generation to another. The cell cycle checkpoints often give signals to the cells when there is an error in replication so as to alt the process for proper repairs. However, when the proteins involved have been disrupted by NPs interaction, there could be further damage to the genetic material and the cell leading to genetic instability. The disturbance of cell cycle checkpoint functions *was also investigated by* Huang *et al.* (2009). They observed that TiO₂ NPs inhibited the mitotic checkpoint PLK1 protein which is involved in controlling numerous processes during

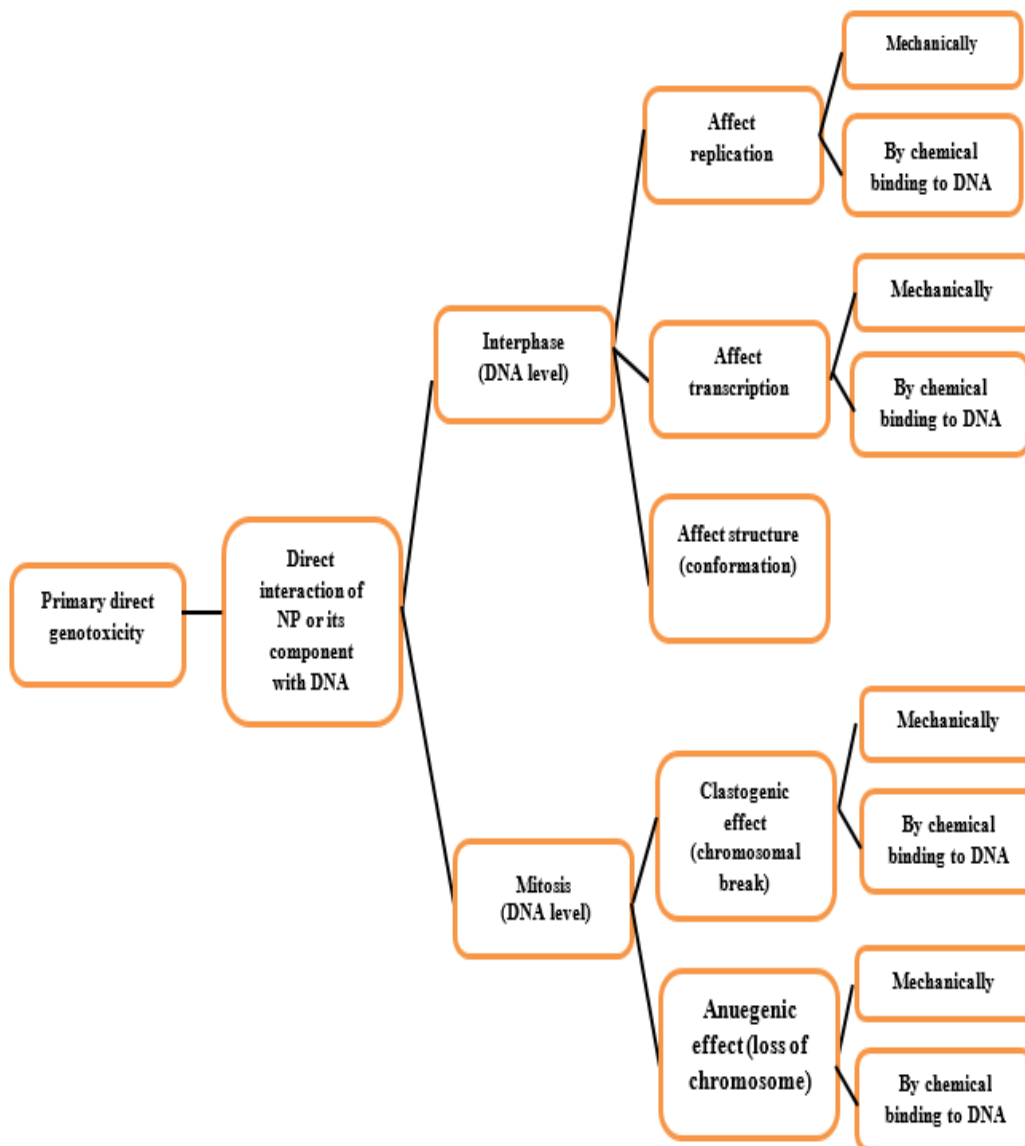


Figure 2.6. Possible mechanism of primary direct genotoxicity

Source: Magdolenova *et al.* (2014)

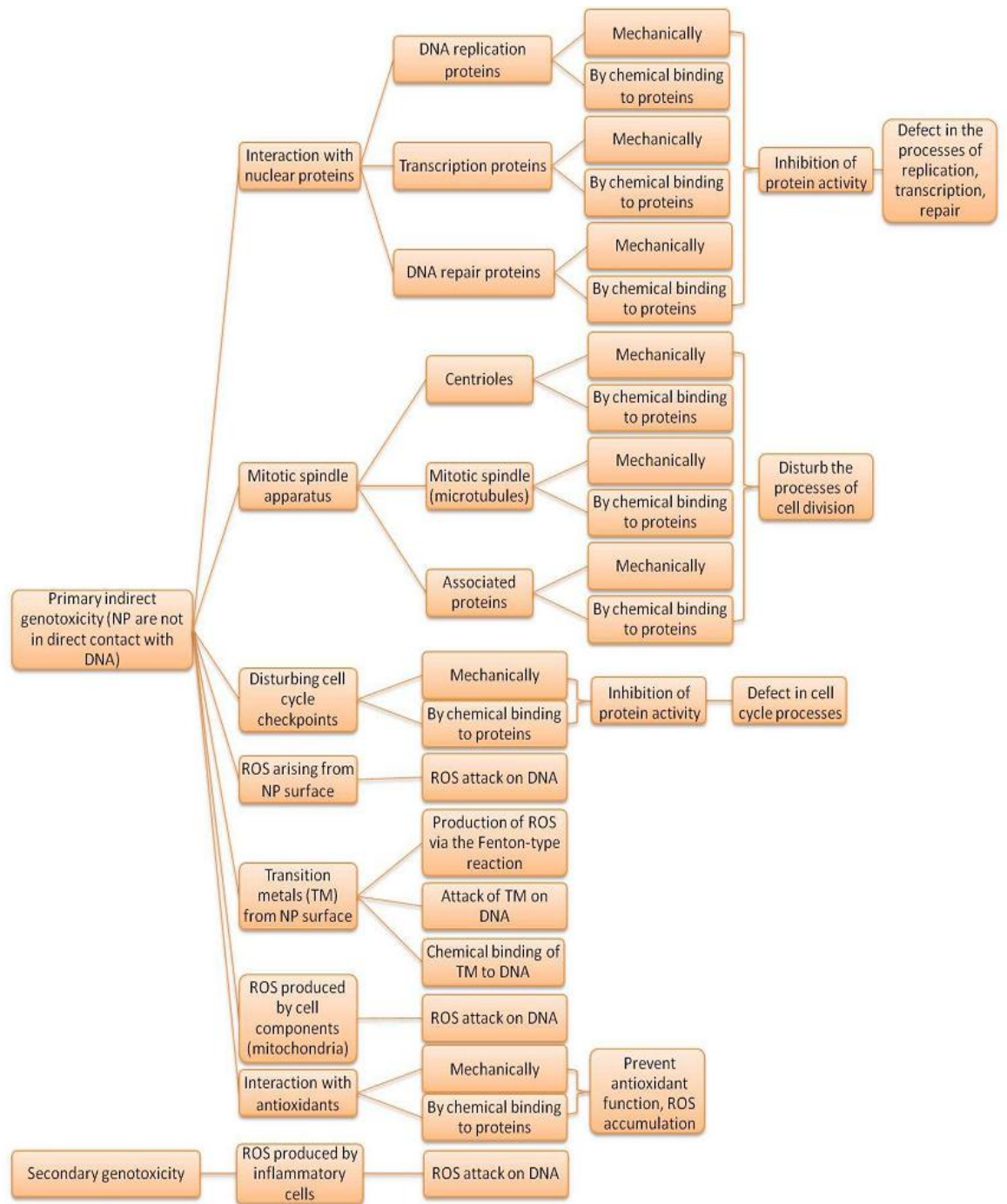


Figure 2.7. Possible mechanism of NP induced genotoxicity (primary indirect and secondary)

Source: Magdolenova *et al.* (2014)

mitosis. One of such consequences is aneuploid or multinucleated cells which can arise from disturbance of cytokinesis. Another important protein involved in cell cycle regulation is kinases which are inactivated by process characterised by polyubiquitin. Findings of Calzolari *et al.* (2010).

Direct production of ROS from NPs surface had also been identified as NPs are capable of generating a free radical attack. Shukla *et al.* (2011) found that TiO₂ NPs elicited free radicals in aqueous suspensions *in vitro*. When ROS interact cellular macromolecules such as DNA, it leads to serious damage (Cooke *et al.*, 2003)

ROS production by mitochondria, binding of transition metals from NP surface, and inhibition of synthesis or activities of anti-oxidant molecules or enzymes are other interactions or activities that could lead to induction of genotoxicity by NPs in the biological systems (Sharma *et al.* 2012).

2.9.2 Secondary genotoxicity

The presence NPs in the biological system could lead to activation of phagocytes (macrophages and neutrophils) which consequently often lead to the production of ROS which if not overcome by the cells endogenous mechanism could lead to DNA damage. Trouiller *et al.* (2009) discovered that TiO₂ NPs elicited oxidative genetic damage and inflammatory responses in mice. They further explained that ROS generated from phagocytic activities may be responsible genotoxic effects. Also, when genetic damage is not properly repaired it might end up in further damage or mutation. These arise when there is extensive damage or impairment of the DNA repair system and hence damage persist (Huang *et al.* 2009). The accumulation of these malformations in the biological system may finally lead to apoptosis and uncontrolled proliferation of cells.

2.10 Review of methods

Genotoxicity assays are vital tools in genetic toxicology to investigate damaging effects of physical and chemical agents when they interact with the genetic materials (DNA) either directly or indirectly (Brusick, 1980). All genotoxicity assays have a common goal of assessing the potential damaging effect of xenobiotics, chemicals, food items and other materials or agents that we continuously find in our environment

or are exposed to. Genotoxicity assessment is key because heritable changes in genetic materials (mutation) is an initiation step in mutagenesis/ carcinogenesis. Coupled with the myriad of chemicals that are constantly produced by man for a better life, genotoxicity assessment remains germane in the assessment of such chemical/substances for a healthy and safer environment.

There are different assays available *in vitro* and *in vivo* for genetic endpoints. These assays measures damage at the level of the gene, DNA or chromosome. However, no single test can provide detailed information needed to delineate the genotoxic potentials of a chemical/substance. Hence, a battery of assays is usually employed in genotoxicity assessment of chemicals. Genetic toxicology aims at identifying mutagens for hazard identification and characterising dose-response relationships and more recently attention is shifted beyond identification to understanding the mutagenic mechanisms of the substance. Among list of genetic assays, chromosome aberration assay, micronucleus assay, *Allium cepa* assay are important assays that are useful in assessment of genotoxicity in either aquatic or terrestrial environments

2.10.1 *Allium cepa* assay

The use of higher plants bioassays is an internationally acclaimed method for monitoring of environmental toxicity (Fiskejö 1993). They possess properties that distinguishes them and makes them relevant in the environmental biomonitoring. They are used in the assessment of different genetic endpoints such as point mutation, chromosome aberration, and this can be assessed in leaves, pollen and roots (Grant, 1994).

Allium cepa (commonly known as onion) among other commonly used plants has large chromosomes and reduced number ($2n = 16$) (Fiskejö, 1993). *Allium cepa* is can be handled and stored easily. Also, its the root tip cells can be assessed for both macroscopic and microscopic characteristics of toxicity (Odeigah *et al.* (1997). *A. cepa* test has been used in the investigations of environmental pollution (Fiskejö, 1985; Bakare *et al.*, 2000; 2009; 2012) testing crude extracts of cyanobacteria (Laughinghouse, 2007), evaluation of genotoxic potential of medicinal plants, anticancer properties of plant extracts (Oyeyemi and Bakare, 2013; Atoyebi *et al.*,

2015) and even genotoxic potential of some nanoparticles (Kumari *et al.*, 2009; 2011; Pesnya, 2013).

The *A. cepa* assay is simple, very cheap and easy to apply. Furthermore, it shows good correlates well other test carried out in mammalian systems. As a robust assay it consist of genotoxicity and toxicity assessment. This makes it possible for application of its data for extrapolation in animals and diverse plants. The chromosome aberration analysis of the test affords for the assessment of both structural and numerical chromosomal alterations over the course of cell cycle (Fiskesjö, 1993; Grant, 1994; Bakare *et al.*, 2009).

Another advantage of the test system is that the *A. cepa* roots contain oxidase enzyme system that is capable of metabolizing test substances (e. g. polycyclic hydrocarbonates and hence it's sensitivity for pro- mutagens. Interestingly, information on length of time it takes to undergo a cell cycle has been established (16 – 24 h), and the roots grow rapidly with numerous cells. More so, it possesses adaptative properties in different environmental conditions (Matsumoto *et al.*, 2004; 2006). Many division cycles can be monitored within short period. It also assesses mitotic index (MI) which is a function rate of cell division; the MI give information about the number of cells undergoing division and proliferative or inhibitory property of test substance Tedesco and Laughinghouse, 2012).

Since the development of the test by Levan (1930) and its standardization by Fiskesjö (1985) various modification and adaption has been carried out. Rank & Nielsen (1993) adapted the method for evaluation of numerous chemicals and mixtures.

2.10.2 *In vivo* micronucleus assay

The is a reliable test that can identify clastogenic (chromosome-breaking) aneugenic and spindle poisoning potential of a chemical or physical substance. The micronucleus, also known as Howell Jolly body, is an extranuclear chromosomal body formed from either fragmented or whole chromosome which did not make it to the poles timely and so is not part of the daughter nuclei during mitosis (Schmid, 1975).

In vivo assessment of micronucleus frequency is an important test in a collection of assays approved by international regulatory bodies for screening drugs, chemical and

general safety assessment. Micronuclei are formed during the process of erythropoiesis. Its formation occurs in the event of damage to chromosome or spindle fibre dysfunction (Figure 2.8). The chromosome fragment or lag there is seen in the cytoplasm because during the cell division it isn't integrated into the daughter nuclei (Krishna and Hayashi, 2000). In mammalian rodent's bone marrow, the erythrocytes develop into polychromatic erythrocytes and still contains RNA hence it appears blue when stained with Giemsa but the main nucleus undergoes extrusion (Figure 2.9). Over time as the PCE matures it loses RNA, therefore, it matures into normochromatic erythrocytes. If a damage to the genetic material had occurred during cell division, it comes out as a smaller nucleus which is often stained blue inside the cytoplasm of the PCE or NCE. However, in rats, the spleen has a splenic removal capability for damage RBCs which makes it impossible to detect micronucleus in peripheral blood but the mice lack this (Figure 2.9).

Micronucleus assay can be conducted in the living system and outside of the living system in cell cultures using plant or animal as test models. This assay assesses micronuclei induction in the cells of the exposed group of organisms in comparison with the unexposed organisms. Genotoxic potential of NPs using different exposure routes (oral, IP, IV, inhalation) have been reported (Patlolla *et al.*, 2015b; Bakare *et al.*, 2016)

Though the MN assay was originally designed for assessment in rodents bone marrow, it is now being applied in other non-rodent and other tissues and cell apart from the bone marrow (Alsabti And Metcalfe, 1995; Celik *et al.* 2005). The mechanism of formation is same in another organism like fish except that site of erythropoiesis is mainly the kidney and that aquatic vertebrates retain their nucleus in mature erythrocytic stages.

In genotoxicity testing, the mouse bone marrow micronucleus assay is widely used and well validated (Morita *et al.*, 1997). However, the piscine version of the MN assay in fish erythrocytes has been recognized as a sensitive tool for the assessment of genotoxins in the aquatic environment (Alsabti and Metcalfe, 1995; Bolognesi and Hayashi, 2011; Bakare *et al.*, 2013). Different fish species are being used; a common and reliable fish species of the tropic in use is the *Clarias gariepinus* (African mud

catfish). The *in vivo* piscine MN assay has become increasingly accepted for evaluation of potential genotoxicity of chemicals.

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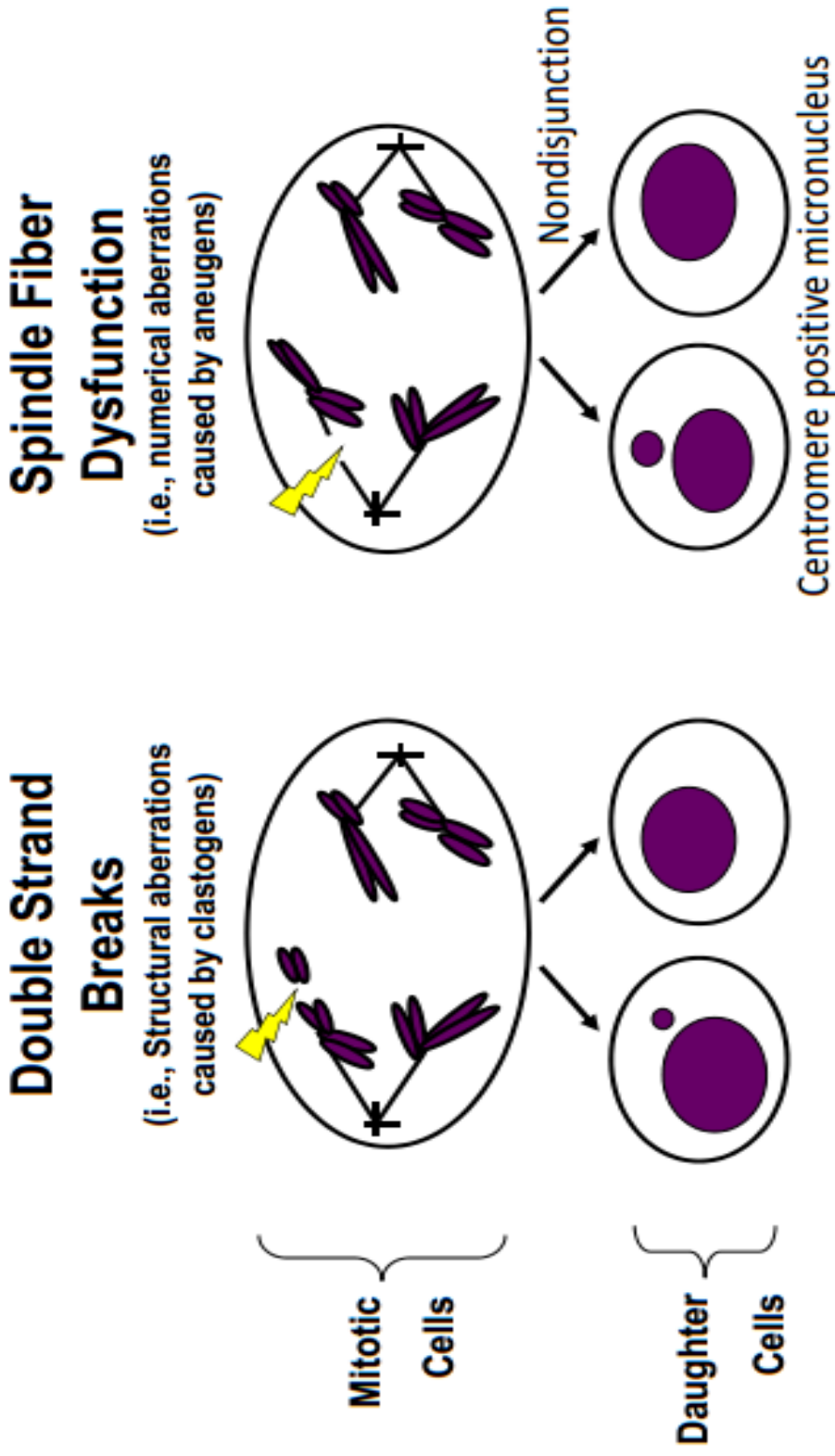


Figure 2.8. Cytogetic damage and formation of micronucleus

Source: White (2017)

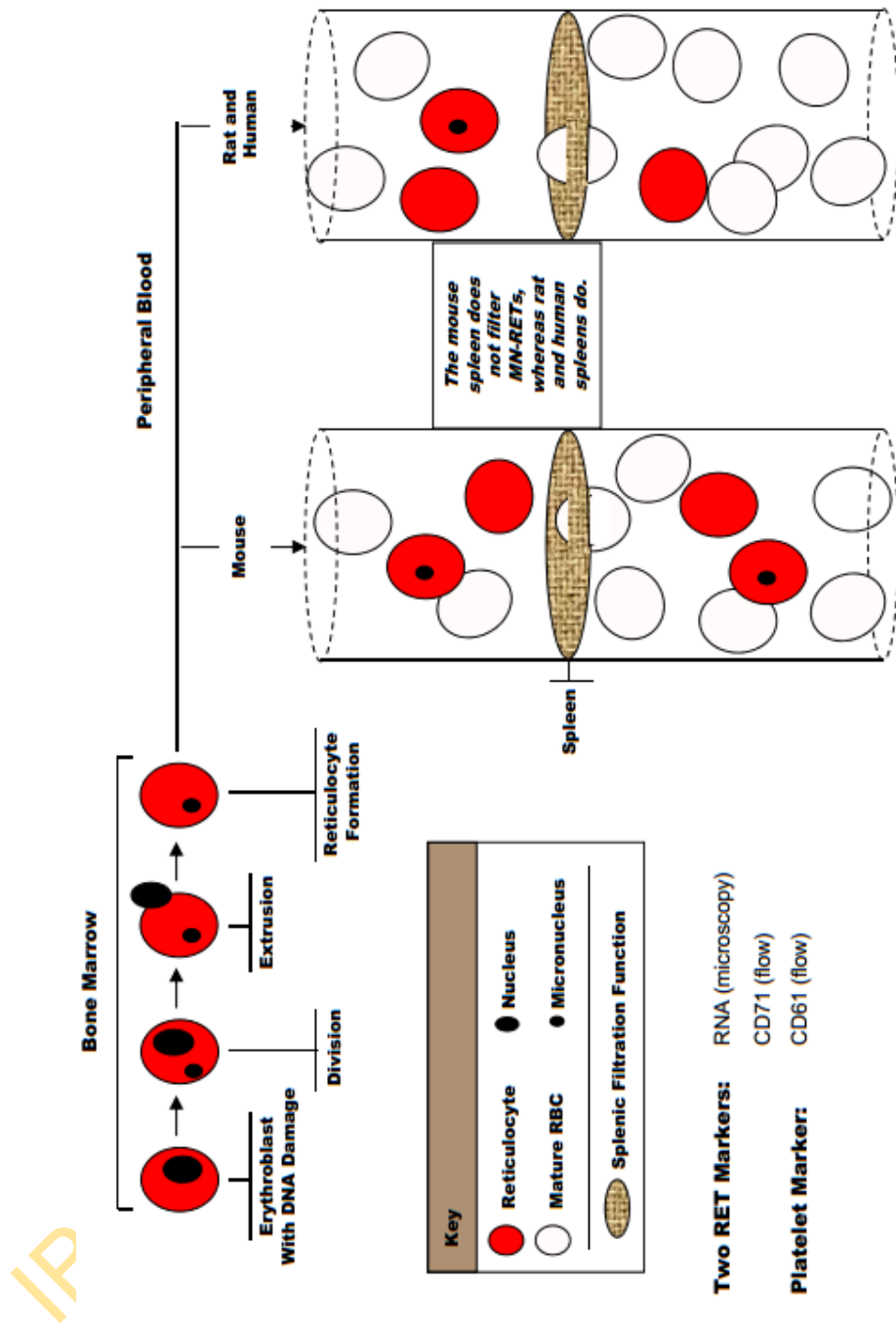


Figure 2.9. *In vivo* microneucleus biology: splenic removal of damage MNPCs in blood
Source: White (2017)

2.10.3 Lipid peroxidation

Lipid Peroxidation is the process by whereby oxidants species destroys lipid membrane, especially the fatty acids which are not saturated (Ayala *et al.*, 2014). It's a widely acclaimed biomarker of oxidative stress. It is often monitored or measured in terms of the level of malondialdehyde (MDA) which is the end product of LPO. The cell puts in place various mechanisms to control lipid peroxidation (Ho *et al.* 2013). In response to membrane lipid peroxidation, the cell may promote cell survival or induce cell death. It is a strong indicator of oxidative damage and consequent genetic damage (Naito *et al.*, 2010)

2.10.4 Anti-oxidant enzyme assays

Metabolic activities in the body system often generates ROS (Reactive Oxygen Species: extremely reactive molecules with lone pair of electrons). These includes superoxide, hydrogen peroxides and hydroxyl radicals, nitric oxide radicals, hypochlorite radical, singlet oxygen. The ROS are can interact with proteins, lipids, carbohydrate and even DNA molecule which could lead to disruption of cellular structures and function (Krishnamurthy and Ashish, 2012). Also, exposure of the living system to xenobiotics often time releases ROS which depends on type and duration of exposure.

In order to combat both endogenous and exogenous production of ROS or lipid peroxidation, the cell activates the production of antioxidant enzymes which scavenges or neutralize ROS. Antioxidant enzymes and molecules include endogenous (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), reduced glutathione (GSH)) and exogenous (tocopherols, ascorbic acid, carotenoids, metal binding proteins, glutathione and lipoic acid). They stabilize the free radicals by donating some of their electrons to complete the lone pair that makes ROS highly reactive (Krishnamurthy and Ashish, 2012).

SOD is involved in the catalytic removal of superoxides by their conversion to hydrogen peroxide as a final product of dismutation Three isoforms of SOD have been identified namely Cu/Zn SOD, Mn SOD and the extracellular SOD (EC SOD). The Cu/Zn SOD is predominantly found in the cytoplasm, nucleus and plasma. While the

Mn SOD is abundant in the mitochondria. The EC SOD is a secretory; in humans there are Mn and Cu - SOD which functions actively in the mitochondria and cytosol respectively; CAT subsequently convert the harmful peroxides to harmless oxygen and water. CAT is an enzyme that consist of 4 identical tetrahedral subunits of 60 KD which enables it to plays tolerance role to oxidative stress for the cell's adaptive responses. GSH P_x also removes hydrogen peroxide by using GSH oxidizing it into oxidized glutathione; glutathione reductase, a flavoprotein (GSSG) generates GSH from GSSG with NADPH as a source of reducing power (Halliwell and Gutteridge, 1999). Reduced glutathione is one the widely available antioxidant molecule in the cell and it is water soluble. It prevents the oxidation of protein thiol groups by either directly reacting with the ROS or indirectly through glutathione transferases (Krishnamurthy and Ashish, 2012).

Reports on NPs toxicity and toxicity of other substance have been used as a mechanistic approach to deciphering role of ROS in toxicity (Bakare *et al.*, 2013; Adeyemi and Faniyan, 2014; Alkaladi *et al.*, 2015)

2.10.5 Serum markers of hepatic and renal injury

The liver and kidney enzyme function test are known markers of damage to liver and kidney in the biological systems; The serum aminotransferases; Aspartase Aminotransferase (ALT) and Aspartase Aminotransferase (AST) are reliable indicators of functional or structural alterations of hepatic cells, and can be useful in the detection of hepatic illnesses (Noguchi *et al.*, 2002; Cogliati *et al.*, 2010). ALT, AST, Alkaline Phosphatase (ALP) and gamma-glutamyltransferase (g-GT), are important metabolic enzymes in liver cells. When the hepatocytes are damaged, they leak into bloodstream, their increased level in the bloodstream is an indication hepatocellular damage. The occurrence of cell death, chemical and drug toxicity often elevate levels of the aminotransferases. Biological activities, chemical and physical factors as well as disturbance in the normal kerb cycle may also alter the activities of these enzymes.

Urea is an end product generated from protein of protein metabolic breakdown in the liver through the urea cycle. It further passes through the filtration process at the glomerulus. In the case of the kidney glomerular compromise, an elevated serum urea

often serves as a biomarker of renal dysfunction which can easily be detected in the blood. However, protein content in diet can affect the production of urea excreted; a diet low or high in protein will either reduce or increase urea level respectively. An increase in serum urea suggest reduction of at least 50% functionality of the kidney. Similarly, the increased presence of serum creatinine is an indicator of renal dysfunction and specifically glomerular dysfunction. Although creatinine is not a toxic substance in the body, its presence due to compromise in glomerular function leads to its increase in bloodstream. Creatinine is a conversion product of creatine. Creatinine is filtered in the glomerulus as such a small amount of it escapes into the tubule and blood stream but in case of damaged glomeruli, the levels rises beyond normal (Zhou *et al.*, 2013).

2.10.6 Histological analysis

Histological assessment is an important aspect of risk assessment and toxicity of foods and chemicals (Yang *et al.* 2017). It provides information on cellular effect of exposure to both acute and chronic exposures of toxic substances in organs/tissues that may not be detected by other biomarkers and correlates well with biochemical analysis and organ weight (Jadhav *et al.*, 2007). A proper and detailed information on histology of affected organs/ tissue will enhance the explanation of the physiological and biochemical processes. Hence, it elucidates structural abnormalities that lead to disorders of function or diseased condition. Toxic substances can cause injuries in internal organs of organisms which might not be visible by mere observing the organism. Analysis of histological structures of organs such as liver, kidney, spleen, gills, brain, have been reported in addition to other toxicity test from various studies in mice, rat and fish. An assessment of silver nanoparticle toxicity showed that it caused morphological alterations in kidneys mice exposed to it (Park *et al.*, 2010). El- Mahdy *et al.* (2015) also reported correlative histopathological effect of AgNPs with other genotoxicity test in rats exposed to AgNPs. Similar findings have also been reported in liver, gill and muscle of fish exposed to silver and copper nanoparticles (Rajkumar *et al.*, 2015; Ostaszewska *et al.*,2016). Histological analysis serve as an indicator of health of organism.

CHAPTER THREE

METHODOLOGY

3.1 Test chemicals

Silver (Ag) and copper(II) oxide (CuO) nanoparticles were purchased from Sigma Aldrich® USA. They possess the following physical characteristics according to the manufacturer: AgNPs: (CAS No.:7440-22-4, <100 nm: 99.5 % metal basis, surface area: 5.0 m²/g , Melting point: 961.93 °C, BP 2212 °C, Relative density: 10.49 g/cm³ and colour: beige to grey) and PVP (Polyvinyl pyrrolidone) a dispersant; CuONPs: (CAS No.:1317-38-0, <50 nm, 99.7 % trace metal basis, surface area 29 m²/g, Melting point: 1326 °C, BP: 2000 °C , Relative density:6.320 g/cm³ and colour: black).

3.1.1 Dispersion of nanoparticles

Nanoparticles were suspended in distilled water and sonicated (Bandelin Sonorex Digitec DT 52H; 35 KHz, 240 W) for 10 mins with 30 secs pause every 3 mins, and vigorously shaken with a vortexing device for 5 mins for uniform distribution of suspension before exposing the test models

3.1.2 Physicochemical characterisation of nanoparticles

The stock solutions for characterisation of the NPs and their 1:1 mixture were prepared by suspending 2 mg of the individual NPs in 1 mL of Milli Q water. The suspensions were ultrasonicated 3 times (3 mins each) with a 30secs pause in between. Then the suspensions were vortexed for 5 mins to obtain a uniform suspension.

Morphologies of the NPs were determined using the TEM according to Georgantzopoulou *et al.* (2016). After suspending the NPs in few mL of ethanol, it was sonicated for a minute and then a drop of the suspension was placed on a conventional TEM Cu mesh grid with amorphous carbon support membrane. The TEM (FEI Tecnai G2 F20) was operated at an accelerating voltage of 120kV. Digital

images were recorded in the bright-field mode using Gatan Ultrascan 2k x 2k CCD camera.

The surface charge and size distribution (hydrodynamic size) of the NPs were characterised using Dynamic Light Scattering (DLS) method. Size distribution and surface charge (zeta potential) of the individual and 1:1 mixture of the NPs in Milli-Q and dechlorinated tap water were measured using Zetasizer Nano series instrument (Malvern Instruments Ltd, UK) according to Cambier *et al.* (2018). The suspensions were measured in triplicates in disposable cuvettes at a temperature of 25°C, and this temperature was actively maintained in the sample chamber. The mean size (hydrodynamic diameter), polydispersity index and the ζ potential were provided by the DLS, with the size distribution regularized by intensity.

3.2 Biological materials

The eukaryotic models used were onion (*Allium cepa* Linnaeus), juveniles of African mud catfish (*Clarias gariepinus* Burchell) and male Swiss albino mice (*Mus musculus* Linnaeus). Onions were purchased from a local Market (Bodija) in Ibadan. They were allowed to dry under sunlight for 3 weeks prior to the commencement of the experiment. Only viable bulbs were used for the study; rotten ones and already growing bulbs were excluded.

Juvenile *C. gariepinus* (25.56 ± 4.79g and SL:13.85 ± 1.06cm) were obtained commercially from the Department of Fisheries, Ministry of Agriculture and Natural Resources, Ibadan, Oyo State Nigeria. The fish were acclimatized for two weeks in transparent plastic aquaria to laboratory condition of 25.5°C, pH 7.2, light and dark photoperiod cycle of 12: 12h at the Departmental Aquatic Toxicology Research Laboratory. Animals were fed with pelletized standard feed.

Male mice (*Mus musculus*), 6-8 weeks purchased from Physiology Department of Physiology, University of Ibadan, were housed at the animal house of Zoology Department, University of Ibadan. The mice were kept at temperature of 27 ± 1°C in plastic cages during acclimatisation (two weeks) and exposure periods and provided with commercial feed pellets (Ladokun pelleted feed®) and drinking *ad libitum*. Both

C. gariepinus and *M. musculus* were cared for and used according to the standard guidelines for handling animals (American Fisheries Society, 2004; ILAR, 2011).

3.3 Chromosome aberration assay in *Allium cepa*

Chromosomal aberration assessment was according to Fiskejö (1985) to study the genotoxic and recovery effect of the nanoparticles and their 1:1 mixture. Five nominal concentrations per NPs and 1:1 mixture (5, 10, 20, 40 and 80 mgL⁻¹) were tested. Eight onion bulbs were used per concentration for both the genotoxicity and recovery assessment of each of the NPs and 1:1 mixture. The dry onions' brown, scales and the dead roots were discarded carefully without destroying the primordial ring of roots. The peeled onions were rinsed in tap water for cleansing and preservation of the roots. The planting of onion bulb was carried out on 100 mL beakers in the dark at room temperature (25 – 27°C). Distilled water and lead nitrate (10 ppm) were used as negative and positive control experiments respectively (Akinboro and Bakare, 2007).

For the genotoxicity experiment, four bulbs were planted in vehicle solution (distilled water) for 48-h period during which the distilled water was changed after 24 h. At 48 h, growing onion bulbs were transferred to the test NPs dispersions. After 24 h, the root cells of the 4 onions were harvested for cytogenetic assessment. For the recovery experiment, another set of 4 onion bulbs that had been grown in test NPs dispersion for 24 h (as was done in the genotoxicity experiment) were transferred back to a beaker of distilled water for another 24 h to check recovery effect from any possible damage. Harvested roots (0.5 – 1 cm) for both genotoxicity and recovery experiments were fixed for 24 h in Carnoy fixative (methanol: glacial acetic acid, 3:1 v/v) and subsequently preserved in 70% methanol until time of use.

The preserved root tips (in 70% ethanol) were hydrolysed with 1N HCl at 60°C for 5 min, rinsed thrice in distilled water and two meristematic root tips were mashed on each slide. The slides were stained for 10 min with acetocarmine. Excessive stain was removed with the aid of Whatman filter paper (150 mm) and a coverslip was carefully used to cover the teased cells. Thereafter, the coverslip was varnished to the microscope slide with nail varnish as described by Grant (1982). A total of 4 slides were prepared per concentration and observed under the microscope for chromosome

aberrations using oil immersion at x1000 magnification. Four thousand (4000) cells were scored per concentration and controls.

3.4 Micronucleus Assay

3.4.1 Acute toxicity test and piscine micronucleus assay

A preliminary range-finding test according to Reish and Oshida (1987) was performed to identify appropriate concentrations for the 96-h acute toxicity test and subsequent sublethal concentration for the genotoxicity test. The range finding test was done at nominal concentrations of 100 mg/L, 500 mg/L of CuONPs and 10 mg/L and 100 mg/L AgNPs under a static bioassay procedure for 96 h. Fish were not fed 24h before and during the test. Each group had 10 fish per replicate in duplicate (EPA, 1996). Fish of average weight 21.73 ± 3.61 g and standard length 14.18 ± 1.53 cm were randomly distributed into 25 L plastic tanks set up in duplicates/group containing either dechlorinated tap water or nanoparticle suspension. The fish were maintained at pH range of 6.8 -7.6. The temperature was within the range of 28.1 – 29.8 °C. Fish showing no opercula movement to tactile stimuli were considered as dead and removed immediately from the media. Behavioural and external morphological changes were also observed.

For the genotoxicity test, five fish randomly selected were exposed to five sublethal concentrations (6.25, 12.5, 25, 50 and 100mg/L) of each of the NPs and their 1:1 mixture for 14 and 28 days in a static renewal bioassay. A maximum threshold concentration of 100 mg/L according to OECD 203 (1992) was considered following an indeterminate acute toxicity test (data not shown). Dechlorinated tap water and benzene (0.05mL/L) served as negative and positive controls respectively. The exposure medium was changed every other day to reduce waste accumulation, remnants of food particles and also maintain the concentration of the NPs dispersion.

The piscine MN assay was done as previously described by Bakare *et al.* (2013). At the end of 14 and 28 - day exposure periods, peripheral blood was drawn from the caudal vein of the fish using needle (25 G) and syringe (2 mL). Thin smear of blood was made on clean microscope slides. The smears were fixed in absolute methanol for a period of 20 min and later stained in 10 % MayGrunwald and 5 % Giemsa stains for

10 and 30 min respectively. Three slides (1,000 erythrocytes per slide) were scored for MN induction at magnification of x 1,000. Other nuclear abnormalities were also scored as biomarkers of cytotoxicity according to Carrasco *et al.* (1990).

3.4.2 Acute toxicity and mouse bone marrow micronucleus assay

The acute toxicity test was performed according to OECD 420 guideline (2001) for estimation of LD₅₀. The oral route of exposure was employed because of potential oral exposure of NPs in the environment. Four mice per group were administered doses of either 300 mgkg⁻¹ bw or 600mgkg⁻¹ bw of each NPs. Food but not water was withheld 4 h before the experiment. The mice were assessed for behavioural signs of toxicity and mortality individually for the first 4 h and then on a daily basis for 14 days. The clinical signs and symptoms of toxicity monitored include vomiting, active movement, active feeding, diarrhoea, mucus secretion, change in fur colour, fur loss and mortality. Individual animal body weight was also monitored daily to observe changes in body weights.

For the genotoxicity test which comprised 2 categories, a total of 136 mice (6 – 8 weeks old) were used. For the first category which was a 14-day exposure experiment, mice were grouped into five groups (corresponding to five concentrations: 18.75, 37.5, 75, 150 and 300mg/kg bw) per NP and their 1:1 mixture. Each group consisting of four (4) mice were repeatedly administered oral doses of 0.2 mL of either AgNPs, CuONPs or their 1:1 mixture for 14 consecutive days at an interval of 24 h. While for second category which was a 28 - day experiment, another set of mice (also five groups per NPs and 1:1 mixture with same concentrations as stated above) were orally administered the NPs for 14 days but left for another 14 days to assess possible recovery effects of any damage or delayed genotoxicity as a result of the oral exposure. Distilled water and Cyclophosphamide (20 mg/kg bw) served as negative and positive controls respectively. Mice were sacrificed 6 h post last dose administration.

Mouse bone marrow micronucleus assay done according to Schmid (1975) and Bakare *et al.*, (2009). Following sacrifice by cervical dislocation, femurs were excised. The epiphyses of the proximal and distal ends of the two femurs were slightly cut with scissors to open up both ends of the bones. Thereafter, the bone marrow was flushed

from each of the bone pair with 1 mL Foetal Bovine Serum (FBS) (Hi Media ® India) into 1.5 mL eppendorf tubes using 1 mL syringe and 25 G needles. The bone marrow cells were mixed and centrifuged twice at 2000 rpm for 5 min. After the first centrifugation, supernatant was discarded and cells re-suspended in another 1mL of FBS before the second centrifugation at the same revolution. The supernatant was discarded and 50 µL FBS was added to the resultant pellet of bone marrow cells and mixed properly to disperse cell clumps. Five slide smears /mouse were made by putting a drop of the cell suspension on one end of the slide and drawing it evenly to the other end. The air – dried slide - smears were fixed in 70% methanol in a couplin jar for 3 min, air –dried and stained with May-Grunwald I and II stains consecutively for 3 min each. Distilled water was used in rinsing the slides thoroughly and counterstained with 5 % Geimsa stain for 5 min, rinsed and air dried. The slides were dipped in xylene. DPX (2-3 drops) were dropped on it and a cover slip was placed on it. One thousand erythrocytes were scored per animal at x1000 for MN in Polychromatic Erythrocytes (PCE) while taking counts of Normochromatic Erythrocytes (NCE) observed alongside 1000 PCE count. Bluish – purple and pinkish – orange colours served as indicator for identification of PCEs and NCEs respectively.

3.5 Systemic toxicity assays

The same set of fish and mice used for the micronucleus assay were used for the systemic toxicity assays in compliance with the 3R principles of reduction of animals used in research.

3.5.1 Blood collection and organ - body weight measurement

Animals were weighed at the beginning and the end of the experiments. At the end of 7 and 28 - day exposure periods of *C. gariepinus* and the 14 and 28 –days for *Mus musculus*, peripheral blood samples were collected from caudal veins and retro – orbital sinus using needle and syringe (25 G;2 mL) and micro – haematocrit capillary tubes respectively. Blood samples from both animals were collected into Ethylene Diamine Tetra Acetic Acid (EDTA) bottles. While another aliquot from mice was collected into plain bottle (for serum biochemical analysis). After sacrifice, liver and gill in fish; and liver, kidney and spleen in mice were excised from individual animals

and weighed. The relative organ weights were calculated and expressed as g/g percent of body weight.

$$\text{Relative organ weight (\%)} = \frac{\text{Absolute organ weight (g)} \times 100}{\text{Final Body weight (g)}}$$

3.5.2 Haematological analysis

Blood collected into EDTA bottles were analysed for haematological parameters such as Packed Cell Volume (PCV), Haemoglobin (Hb), Red blood cell counts (RBC), Total white blood cell count (WBC), white blood differentials (lymphocytes, heterophils, monocytes, eosinophils, basophils) and Platelet counts were analysed using standard procedures. RBC indices such as Mean Cell Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentrations (MCHC) were also calculated (Blaxhall and Daisely, 1973). This analysis was carried out at Haematology Laboratory of the Department of Veterinary Pathology, University of Ibadan.

3.5.3 Serum Biochemistry

Blood collected into plain (eppendorf) tubes was kept at room temperature for 30 min – 1 hour to clot and then centrifuged for 10 min at 3000 rpm. The clear supernatant (serum) was collected and stored at -20°C. The serum was used for the liver and kidney function tests (Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and urea tests). Serum biochemical analysis was carried out using appropriate diagnostic kits (Randox Laboratories, County Antrim, United Kingdom).

3.5.3.1 Determination of Alanine Aminotransferase (ALT) Activity

This activity was carried out according to Reitman and Frankel (1957). Serum ALT activity was determined based on the principle that ALT catalysed reactions between alpha oxoglutarate and L – alanine to produce pyruvate as shown in the equation below. The pyruvate formed is coupled with chromogen solution in an alkaline

medium to form coloured oxaloacetate hydrazone. The intensity of the hydrazone measured using a spectrophotometer is proportional to the activity of alanine aminotransferase.

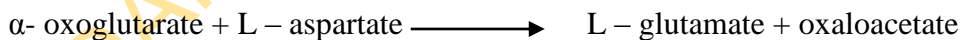


Diluted sample (10 μL) was mixed with 50 μL of the first reagent containing phosphate buffer (100 nM/L, pH 7.4), L-alanine (200 mM/L) and α -oxoglutarate (2 mM/L). The mixture was incubated for 30 min at 37 $^{\circ}\text{C}$. A volume of reagent II (50 μL) was added to the resultant solution and incubated at room temperature for 20 min. Later 500 μL of 0.4 mol per litre NaOH was then added and the absorbance was read against the blank using a spectrophotometer (Jenway 6305, Cole Palmer, UK) after 5 min at 546 nm. The activity of ALT (U/L) in serum was obtained by extrapolation from the standard table provided by the kit manufacturer.

3.5.3.2 Aspartate aminotransferase (AST) activity

Similarly, AST activity was determined according to Reitman and Frankel (1957).

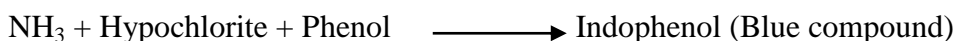
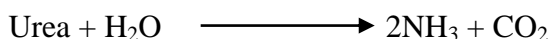
This is based on the principle that oxaloacetate which is formed from the aspartate aminotransferase catalysed reaction between alpha oxoglutarate and aspartate is coupled with chromogen (2, 4 – dinitrophenyl hydrazine) in an alkaline medium to form oxaloacetate hydrazone. The intensity of the coloured hydrazone formed is proportional to the aspartate aminotransferase activity.



Diluted sample (10 μL) was added to 50 μL of reagent I containing phosphate buffer (100 mM/L, pH 7.4), L- aspartate (100 mM/L) and α -oxoglutarate (2 mM/L). The mixture was incubated for 30 min at 37 $^{\circ}\text{C}$. Then, 50 μL reagent II was added to the reaction mixture and incubated at room temperature for 20 min. 500 μL of 0.4 mol/L NaOH was added and the absorbance was read after 5 min at 546 nm wavelength. Activity of AST in serum was obtained from a standard curve provided by the kit manufacturer.

3.5.3.3 Urea concentration

Serum urea was determined as described by Fawcett and Chemes (1979). This is based on the principle that serum urea undergoes to form ammonia in a reaction catalysed by urease. The following equation which involves the conversion of the ammonia produced to indophenol underlies the method.



Using the Randox kit protocol, 10 μL of sample was added to 100 μL of reagent I (containing sodium nitroprusside and urease). The solution was mixed and incubated for 10 min at 37°C. Furthermore, 2.5 mL each of phenol and sodium hypochlorite were added, mixed and subsequently incubated for 15 mins at 37°C. Absorbance of sample and standard was measured against blank. Urea concentration was calculated as follows

$$\frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{Standard concentration}$$

Where A_{sample} absorbance reading for the sample and A_{standard} for the standard

3.5.4 Oxidative stress analysis

Liver samples excised from *C. gariepinus* (after 28 days) and *Mus musculus* (after 14 and 28 days) exposure periods were rinsed in 1.15% KCl, blotted, weighted and stored in 50 mM phosphate buffer at -20°C, till time of analysis. Samples were thawed, homogenised in 4 volumes of 50 mM Phosphate buffer (pH 7.4) using tissue homogeniser. The resulting homogenate was spun at 10,000 rpm at 4°C for 10 min. The supernatant, was preserved at -20°C until use. Subsequently, the homogenate was used for the assay of hepatic antioxidant enzymes.

3.5.4.1 Total Protein content determination

Protein concentration of the samples was determined using the Biuret method (Gornal *et al.*, 1949) with slight modification. Potassium iodide (KI) was added to biuret reagent to prevent the precipitation of Copper II ion (Cu^{2+}). It is based on the ability of proteins form a coloured complex in the presence of copper ion in a basic solution.

Biuret reagent used contained copper tetraoxosulphate (VI), potassium iodide and sodium potassium tartarate.

The samples were diluted 5 times with distilled water (0.2 mL of samples into 0.8 mL of distilled water). Biuret reagent (1.5 mL) was pipetted into test tube containing 100 μ L of the sample, incubated for 30 min at room temperature and spectrophotometer reading was taken at 540 nm. Standard Albumin curve was used to extrapolate the protein content.

3.5.4.2 Estimation of Lipid peroxidation

Lipid peroxidation was carried out as described by Rice-Evans *et al.*, (1986). This is based on ability of the reaction of 2-thiobarbituric acid (TBA) and the end product formed from lipid peroxidation (Malondialdehyde -MDA) which forms a pink coloured compound when heated in an acidic medium.

An Aliquot of 400 μ L of the sample was mixed with 1600 microliters of Tris-KCl buffer (containing 500 μ L of 30 % TCA). Then 500 μ L of 0.75 % TBA was added to the reaction mixture and heated at 80⁰C for 45 min, cooled and spun in the centrifuge at 3000 rpm for 15 sec. The resultant solution was allowed to cool on ice and then measured spectrophotometrically at 532 nm against a reference blank of distilled water. Lipid peroxidation in units /mg protein or gram tissue was computed with a molar extinction coefficient of $1.56 \times 10^5 \text{M}^{-1}\text{Cm}^{-1}$.

$$\text{Malondialdehyde level} = \frac{\text{Absorbance X volume of mixture}}{E_{532 \text{ nm}} \text{ X Volume of sample x mg protein}}$$

3.5.4.3 Superoxide Dismutase Assay (SOD)

The level of SOD was determined by the method of Mistrá and Fridovich (1972). The ability of SOD to inhibit the autoxidation of epinephrine at pH 10.2 makes this reaction a basis for a simple assay for SOD. Superoxide radical generated by the xanthine oxidase reaction caused the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per superoxide molecules introduced increased with increasing pH and increased with increasing concentration of epinephrine. This leads to the autoxidation of epinephrine which proceeds by at least two distinct

pathways, one of which is free radical chain reaction involving superoxide radical and hence inhibitable by SOD.

One mL of sample was diluted in 9 mL of distilled water to make a 1:10 dilution. An aliquot of 0.2 mL of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2). Then 0.3 mL of freshly prepared adrenaline was added to the mixture and quickly mixed by inversion. The reference cuvette contained 2.5 mL carbonate buffer, 0.3 mL of adrenaline and 0.2 mL of distilled water. The absorbance was taken every 30 sec for 150 sec. Absorbance rate was determined as follows

$$\text{Increase in absorbance per minute} = \frac{A_{480} \text{ at } 2:30 - A_{480} \text{ at } 0:00}{2.5}$$

Where A_{480} at 0.00 = absorbance at 0 sec

A_{480} at 2:30 = absorbance at 150 sec

Determination of % inhibition for the test sample

$$\% \text{ Inhibition} = \frac{\text{increase in absorbance of substrate}}{\text{increase in absorbance of blank}} \times \frac{100}{1}$$

1 unit of SOD activity is the amount of SOD necessary to cause 50% inhibition of oxidation of adrenaline.

3.5.4.4 Catalase (CAT) Activity

Catalase activity was determined according to Claiborne (1985). This is based on the absorbance loss at 240 nm wavelength when catalase splits hydrogen peroxide. An extinction coefficient of $0.0436 \text{ mM}^{-1}\text{cm}^{-1}$ was used for its estimation.

Hydrogen peroxide (2.95 mL of 19 mM solution) was pipetted into a 1 cm quartz cuvette and 50 μL of sample was added. The mixture was rapidly inverted to mix and then placed in a spectrophotometer. Change in absorbance was read at 240 nm every 15 sec for 1 min.

$$\begin{aligned} \text{Catalase activity} &= \frac{\Delta 240/\text{min} \times \text{reaction volume} \times \text{dilution factor}}{0.0436 \times \text{sample volume} \times \text{mg protein/mL}} \\ &= \mu\text{mole H}_2\text{O}_2/\text{min/mg protein} \end{aligned}$$

3.5.4.5 Estimation of reduced glutathione (GSH) level

This was carried out according to the method of Beutler *et al.* (1963). The reduced form of glutathione comprises in most instances the bulk of cellular non-protein sulfhydryl groups. This method is therefore based upon the development of a relatively stable (yellow) colour when 5', 5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of Ellman's reagent with the reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a molar absorption at 412nm. The absorbance of this complex at 412nm is proportional to the level of reduced glutathione in the test sample.

Test sample (0.1 mL) was diluted in 0.9 mL distilled water to give a 1:10 dilutions. Three mL of 4% sulphosalicylic acid solution was added to the diluted test sample to deproteinise it. The mixture was centrifuged at 3000 rpm for 10 min. Thereafter, 4.5 mL of DTNB was added to 0.5mL of the supernatant. A blank was prepared with the reaction mixture of 0.1M phosphate buffer, 0.5 mL of the diluted precipitating solution (addition of 3 mL of precipitating solution plus 2 mL of distilled water) and 4.5 mL of DTNB. All readings were taken within 5 min at 412 nm, since colour developed is not stable. Reduced glutathione, GSH, is proportional to the absorbance at 412 nm.

3.5.5 Histological analysis

Sections of skin, gill and liver samples of fish used for MN assay were excised following sacrifice. These samples were rinsed in phosphate buffered saline, blotted and fixed in Bouin's fluid. They were further treated by dehydrating in ascending grades of ethyl alcohol (70, 90, 95 and 100 %) and cleared using xylene. The sections were impregnated and embedded in paraffin wax using Leica Histokinette tissue processor. A small section of 4µm thickness was processed on glass slides using a rotary microtome, stained with haematoxylin and eosin, then mounted and viewed at x 100 and x 400 for the presence of lesions /morphological disruptions.

3.6 Precautions taken during the course of the experiment

In handling the nanoparticles, the following precautions were taken to avoid spillage and contamination to reduce potential exposure. These included:

- nanoparticles were transported in closed containers in between laboratories and animal facility.
- wearing of laboratory coats, nitrile gloves and thick hand gloves, eye goggles, and nose masks.
- used contaminated nitrile gloves and plastics were kept in biohazard bags until disposal.
- nanoparticles containing containers were properly labelled to avoid mix-ups and contaminations.
- proper hand washing was performed after handling nanoparticles.
- fume hood work benches were wet-wiped after each use or at the end of the day.
- movement was restricted in the laboratory where the aquatic experiment was carried out
- carcasses of animal models exposed to nanoparticles were buried in the soil.

3.7 Ethical guidelines

Institutional ethical approval was obtained for the study prior to start of the experiment (Approval number = ACUREC/APP/2015005) (Appendix I).

3.8 Statistical analysis

For the chromosome aberration assay in *Allium cepa*, the mitotic index was calculated by counting the number of dividing cells per 4000 cells as follows:

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells scored}} \times \frac{1000}{1}$$

Mitotic inhibition was computed as

$$\frac{\text{Mitotic index in control} - \text{Mitotic index in treatment}}{\text{Mitotic index in control}} \times \frac{100}{1}$$

(Akinboro and Bakare, 2007)

For the piscine micronucleus experiment,

$$\text{MN/NA Frequency per 100 erythrocytes} = \frac{\text{No of cells with MN or NAs}}{\text{Total no of cells counted}} \times 1000$$

The percentage polychromatic erythrocyte (%PCE) was computed as

$$\frac{\text{Total number of PCE}}{\text{Total number of PCE} + \text{Total number of NCE}} \times \frac{100}{1}$$

(Alabi and Bakare, 2011; Bakare *et al.*, 2013)

GraphPad prism 5.0 was used for data analysis. Statistical comparison was done using the one-way analysis of variance (ANOVA) followed by Dunett post hoc test at 0.05 probability level. Values are expressed as Mean \pm SE. The two-way analysis of variance was also used to compare the effects of concentrations and exposure periods on genotoxic responses .

Interaction factor analysis of AgNPs and CuONPs mixture on MN induction and Oxidative stress parameters were calculated as described by Katsfis *et al.* (1996).

$$\begin{aligned} \text{Interaction Factor (IF)} &= (\text{AC} - \text{Control}) - [(\text{A} - \text{Control}) + (\text{C} - \text{Control})] \\ &= \text{AC} - \text{A} - \text{C} + \text{Control} \end{aligned}$$

$$\text{SE}_{\text{IF}} = \sqrt{(\text{SE}_{\text{AC}})^2 + (\text{SE}_{\text{A}})^2 + (\text{SE}_{\text{C}})^2 + (\text{SE}_{\text{Control}})^2}$$

Where A and C is in this case, were the mean responses to AgNPs and CuONPs respectively. AC was the mean response of the 1: 1 mixture of the NPs. SE_{IF} denotes

the standard error of interaction factor and SE_i is the standard error of the mean for the respective group i

CHAPTER FOUR

RESULTS

4.1 Characterisation of silver, copper(II) oxide nanoparticles and their 1:1 mixture

The transmission electron microscope images of Ag and CuO nanoparticles (Figures 4.1 and 4.2) showed that both NPs were round and has particle sizes less than 100 nm. Figures 4.3 and 4.4 shows the size distribution by intensity and surface charge respectively for both NPs and their 1:1 mixture's stock suspension in Milli Q and dechlorinated tap water. The Z -average hydrodynamic diameter of Ag nanoparticles in Milli Q water and dechlorinated tap water of 219.4 nm and 318.9 nm respectively shows moderate agglomeration. The mean hydrodynamic diameter of CuONPs showed high agglomeration with 2,260 and 2,222 nm Z – average sizes in Milli Q water and the dechlorinated tap water respectively. While diameters of 282.5 nm and 455.5 nm (of moderate agglomeration) were recorded as Z – averages for the 1:1 mixture in Milli Q and dechlorinated tap water respectively; the polydispersity index of the NPs and their 1:1 mixture in both Milli - Q and dechlorinated tap water were within mid-range values of 0.42 – 0.80 (Figure 4.3; Table 4.1). The surface charge (zeta potential) of AgNPs in Milli Q water (-25 ± 5.55 mV) was lower than the value recorded in dechlorinated water (-17 ± 86.3 mV). A zeta potential (ζ) of 5.29 ± 4.58 and -14 ± 99.73 mV were recorded for the CuONPs while -16.4 ± 6.76 mV and -

15.5 ± 71.5 mV were recorded for the 1:1 mixture in both Milli- Q and dechlorinated tap water.

4.2 Chromosome aberration induced in the meristematic root cells of *Allium cepa* by silver, copper(II) oxide nanoparticles and their 1:1 mixture

Macroscopic examination of the *A. cepa* roots at post-harvest following 24 h exposure and recovery periods, revealed a colour change from white to light yellow (Figure 4.5) compared to the negative control group (Distilled water). Tables 4.2 and 4.3 shows the indices of mitotic activities and chromosome aberration (CA) induced in *A. cepa* roots cells following 24 h exposure to AgNPs and the effect of 24 h recovery period in distilled

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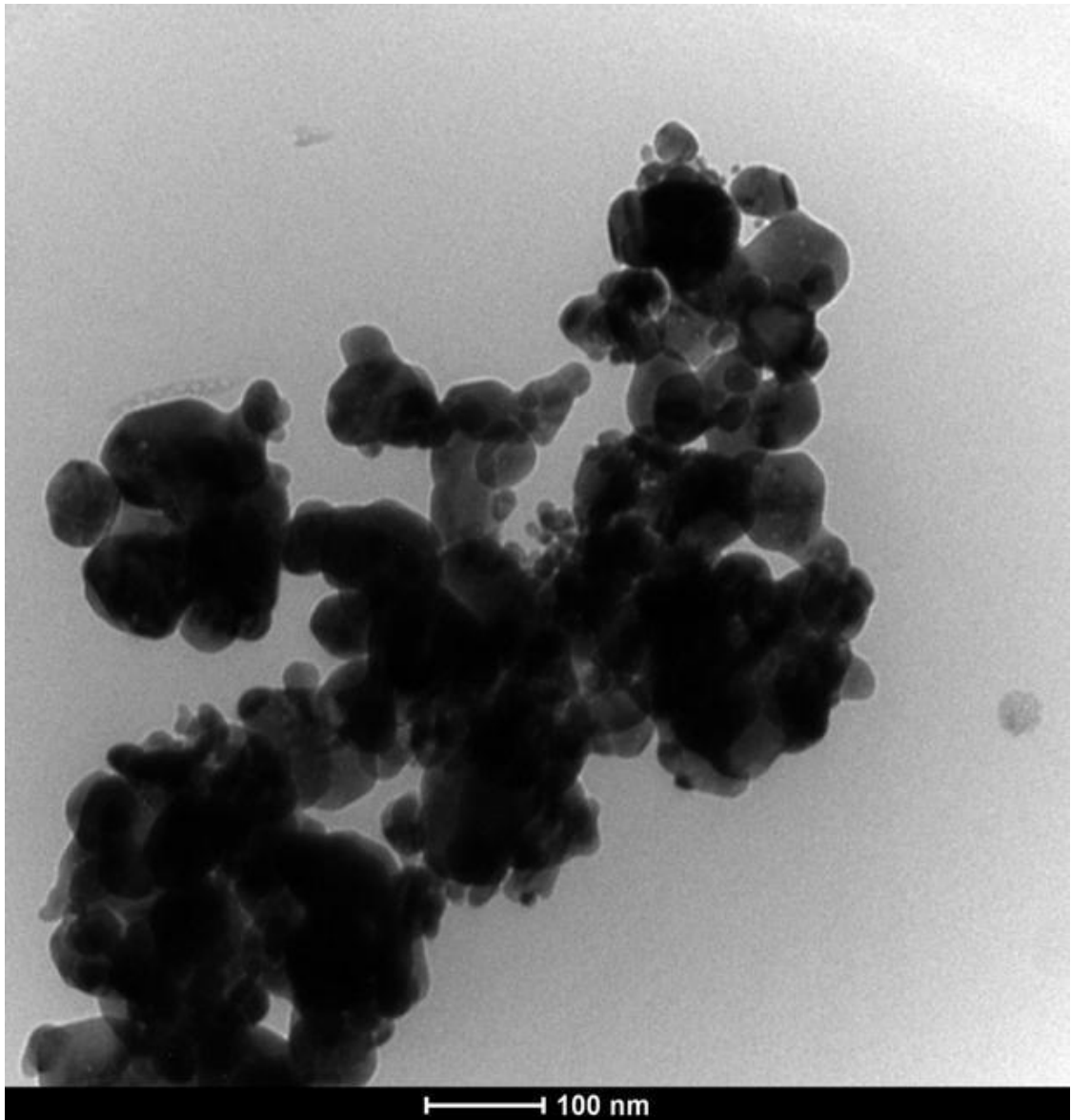


Figure 4.1. Transmission electron microscope image of Ag nanoparticles

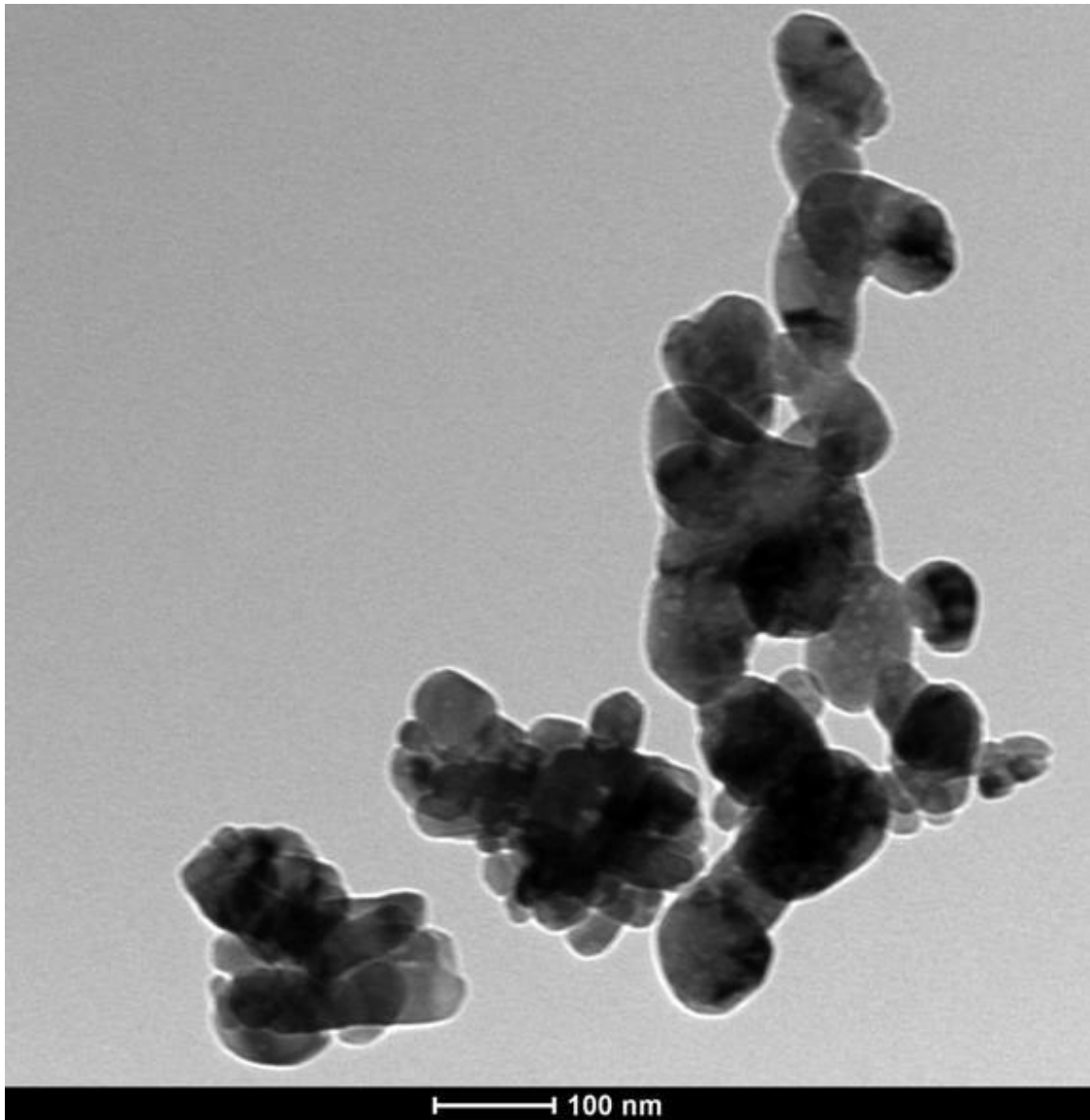


Figure 4.2. Transmission electron microscope image of CuO nanoparticles

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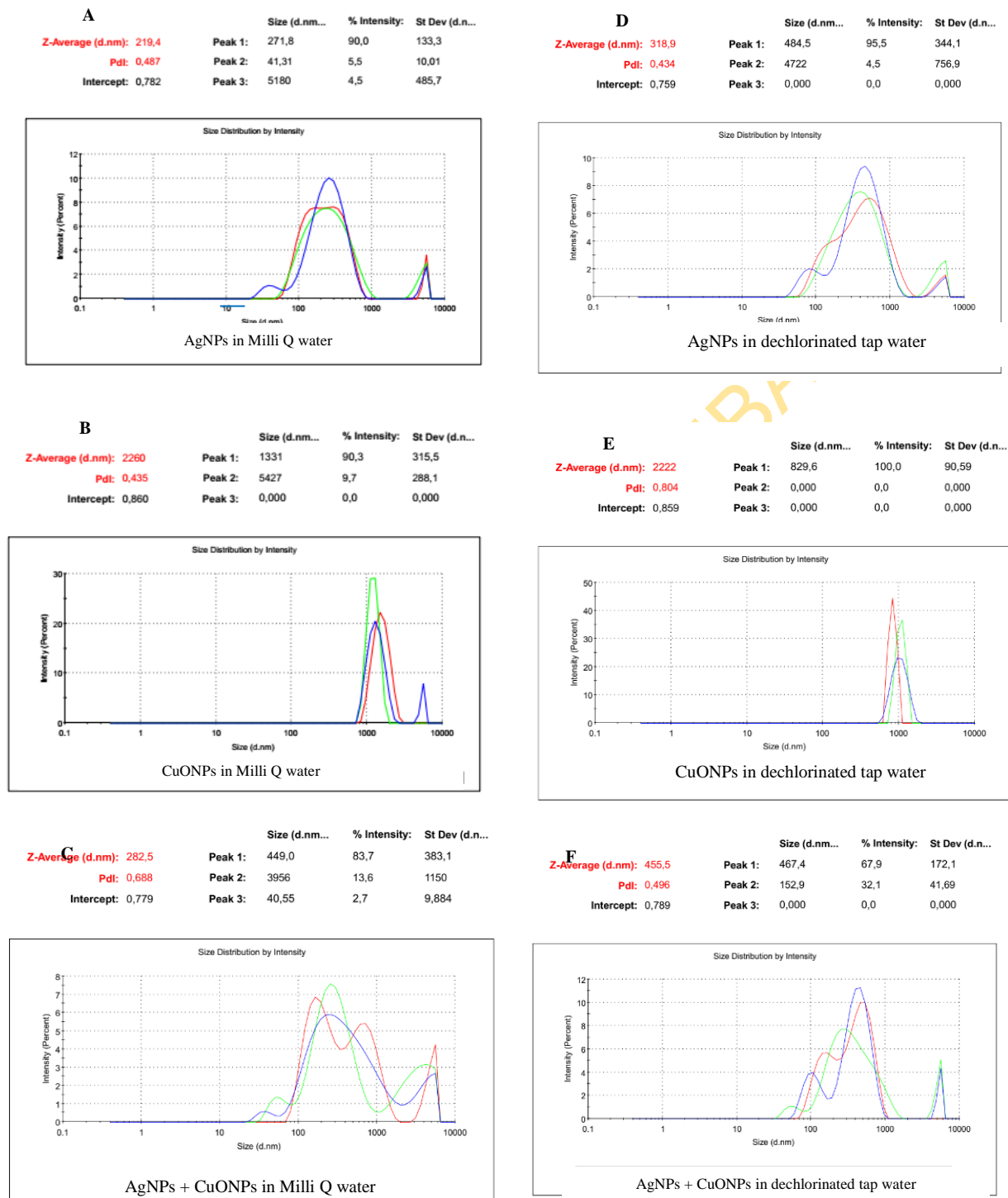
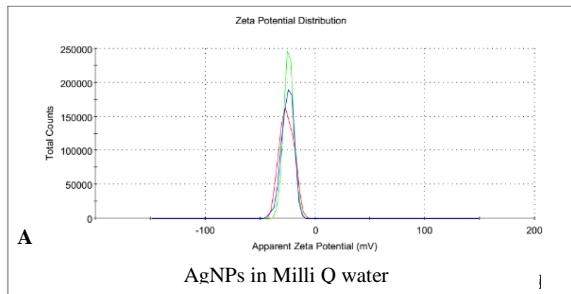
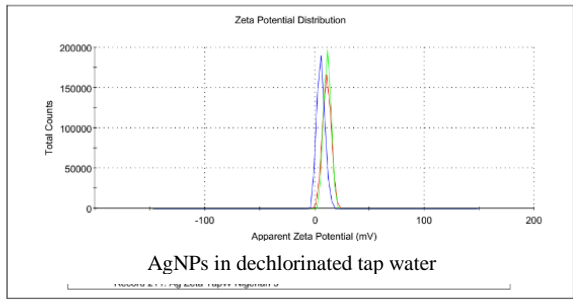


Figure 4.3. Size distribution of AgNPs, CuONPs and their 1:1 mixture measured by dynamic light scattering in milli Q (A-C) and dechlorinated tap water (D- F)

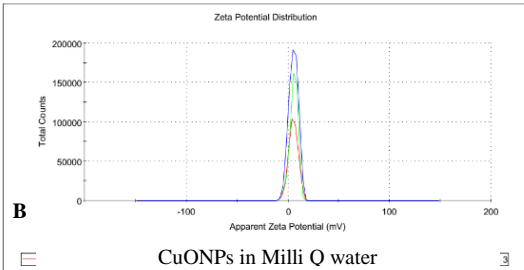
	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -25,3	Peak 1: -25,3	100,0	5,55
Zeta Deviation (mV): 5,55	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,00146	Peak 3: 0,00	0,0	0,00



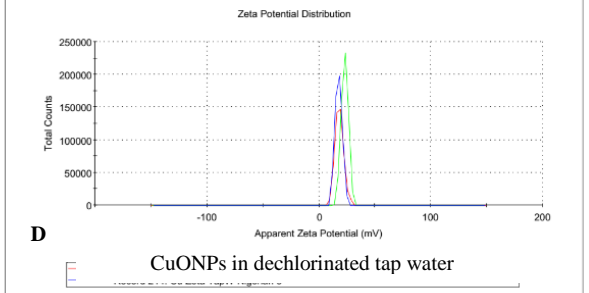
	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -17,0	Peak 1: 11,0	100,0	3,86
Zeta Deviation (mV): 86,3	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,496	Peak 3: 0,00	0,0	0,00



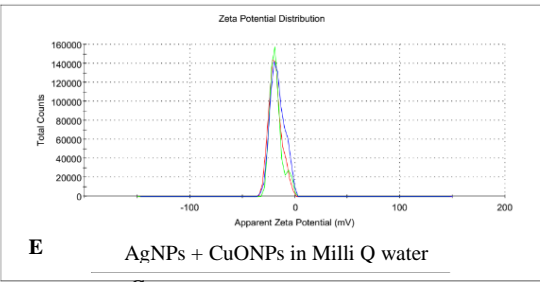
	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): 5,29	Peak 1: 5,29	100,0	4,58
Zeta Deviation (mV): 4,58	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,0210	Peak 3: 0,00	0,0	0,00



	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -14,0	Peak 1: 18,1	100,0	3,85
Zeta Deviation (mV): 97,3	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,494	Peak 3: 0,00	0,0	0,00



	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -16,4	Peak 1: -16,4	100,0	6,76
Zeta Deviation (mV): 6,76	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,00294	Peak 3: 0,00	0,0	0,00



	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -15,5	Peak 1: 8,51	100,0	3,67
Zeta Deviation (mV): 71,5	Peak 2: 0,00	0,0	0,00
Conductivity (mS/cm): 0,486	Peak 3: 0,00	0,0	0,00

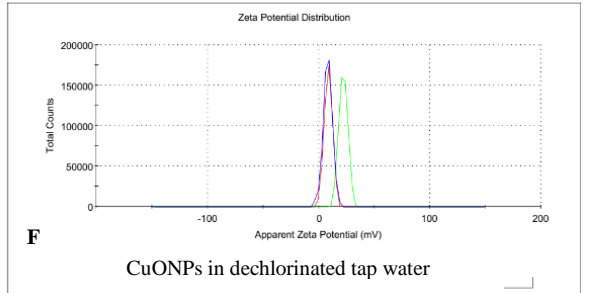


Figure 4. 4. Zeta potentials of AgNPs, CuONPs and their 1:1 mixture measured by dynamic light scattering in milli Q water (A-C) and dechlorinated tap water (D- F)

Table 4.1 Dynamic Light Scattering (DLS) analysis of silver and copper(II) oxide Nanoparticles in Milli Q and dechlorinated tap water

Nanoparticles	Z – Average size (nm)		Polydispersity Index		Zeta potential (mV)	
	Milli Q	Dechlorinated tap water	Milli Q	Dechlorinated tap water	Milli Q	Dechlorinated tap water
AgNPs	219.40	318.90	0.49	0.43	-25.30	-17
CuONPs	2260	2222	0.44	0.80	5.29	-14
Mixture	282.50	455.50	0.69	0.50	-16.40	-15.50



Figure 4.5. Typical macroscopic effect of the nanoparticles on *Allium cepa* roots. (a) Adsorption of NPs on roots (b) Observed change in colour of root from white to light yellow at point of harvest

water (which was the medium for the NPs dispersion). The mitotic index (MI) of the *A. cepa* root cells in the negative control group at 72 h and 96 h were 47.00 and 39.50 respectively while 24 h exposure to positive control (lead nitrate) presented with an MI of 32.80 (at 72 h) and increased to 40.00 following transfer back into the recovery solution (at 96 h). A significant increase ($p < 0.05$) in the MI (Table 4.2) was also observed in the *A. cepa* root cells exposed to AgNPs and the recovery group. At the tested concentrations: 5, 10, 20, 40 and 80 mg/L, the MI of the AgNPs exposed cells were 72.00, 73.80, 61.50, 68.00 and 70.80; and the MI for the corresponding recovery groups were 85.00, 58.30, 88.00, 46.00 and 48.50 respectively.

AgNPs significantly ($p < 0.05$) induced CA in *A. cepa* root cells following 24 h exposure and recovery effect (Table 4.3). At 72 h. The lowest induction of CA in total scored cells (0.43%) was observed at the least concentration of 5 mgL⁻¹. Percentage (%) frequency of CA induced at 10, 20, 40 and 80 mgL⁻¹ were 0.83, 1.13, 0.83 and 1.10 % respectively. The % frequency of CA also increased in the recovery experiment (at 96 h) compared to the corresponding control. However, the *A. cepa* cells recovered partially from damage in the previous mitotic cycle with the total % frequency of CA induced at 96 h being generally lower compared (2.77%) to the total CA induced at 72 h (4.32%).

There was a significant decrease ($p < 0.05 - 0.001$) in the MI observed in *A. cepa* root cells exposed to CuONPs (Table 4.4) from 10 mgL⁻¹ to 80 mgL⁻¹ concentration. The mitotic inhibition recorded in the *A. cepa* root cells exposed to CuONPs at 5, 10, 20, 40 and 80 mgL⁻¹ were 5.85, 79.79, 100, 100, and 94.10 % respectively. Similar decrease in MI was observed following 24 h recovery but was not statistically significant ($p > 0.05$) compared to the control. The CuONPs exposure led to complete cell arrest at interphase at 20 and 40 mgL⁻¹ and a very large proportion of cells observed in both exposure and recovery groups were at the interphase stage with a little proportion of dividing cells (Table 4.5). There was no significant induction of CA increase in the few dividing cells exposed to CuONPs at 5 and 10 mgL⁻¹. However, there was a significant induction of CA based on dividing cells at all tested concentrations. The CuONPs exposed cells recovered from cell arrest but not CA. The percentage frequency of CA in dividing cells in the recovery solution were higher than those of the corresponding exposure groups (Table 4.5).

Table 4.2. Mitotic activities of *A. cepa* grown in AgNPs following 24 h exposure and 24 h recovery periods

Conc. (mg/L)	Effects of 24 h exposure			Effect of 24 h recovery		
	Number of dividing cells	Mitotic index	Mitotic inhibition	Number of dividing cells	Mitotic index	Mitotic inhibition
NC	188	47.00	0.00	158	39.50	0.00
PC	131	32.80	30.32	160	40.00	-1.27
5	288	72.00*	-53.19	340	85.00*	-115.19
10	295	73.80*	-56.91	233	58.30	-47.47
20	246	61.50	-30.85	352	88.00*	-122.79
40	252	63.00	-34.04	186	46.50	-17.72
80	283	70.80*	-50.53	194	48.50	-22.79

Conc: Concentration; NC: Negative control (Distilled water)

PC: Positive control 10 ppm Pb(NO₃)₂. Lead nitrate

*Values significant at p < 0.05

Number of cells scored per concentration = 4000

Table 4.3. Cytological effects of AgNPs on *A. cepa* root tips following 24 h exposure and 24 h recovery periods

Conc. (mg/L)	Mitosis					Chromosome abnormalities										Frequency (%) of aberrant cells based on:		
	No. of dividing cells	No of cells at prophase	No of cells at metaphase	No of cells at anaphase	No of cells at telophase	Sticky chromosome	Anaphase bridge	Distributed metaphase	Spindle disturbance	C – mitosis	Vagrant chromosome	Pole deviation	Binucleated cells	Micronucleated cells	Fragmented nuclear	Total aberrant cells	Total cells scored	No. of dividing cells
24 h exposure period																		
NC	188	140	17	15	16	1	-	4	-	-	-	-	-	-	-	5	0.13	2.66
PC	131	94	12	23	2	24	-	1	5	-	-	-	1	-	-	31	0.78	23.66**
5	288	204	32	14	38	9	-	1	3	-	-	-	4	-	-	17	0.43*	5.90
10	295	209	34	20	32	13	4	4	5	2	-	-	4	1	-	33	0.83*	11.19
20	246	161	36	12	37	14	1	3	14	5	3	1	4	-	-	45	1.13*	18.29
40	252	177	25	26	24	6	3	4	13	-	-	6	1	-	-	33	0.83*	13.10
80	283	179	42	23	39	18	2	1	15	-	2	3	3	-	-	44	1.10*	15.55
Total	1364	930	169	95	170	60	10	13	50	7	5	10	16	1	-	172	4.32	64.03
% Freq		68.18	12.39	6.96	12.46	34.88	5.81	7.56	29.07	4.07	2.91	5.81	9.30	0.58	0	100		
24 h recovery period																		
NC	158	142	5	8	3	8	-	-	-	1	-	-	-	-	-	9	0.23	5.70
PC	160	102	23	24	11	12	1	1	5	5	-	-	-	-	-	24	0.6	15.00**
5	340	267	18	27	28	12	1	-	8	5	-	5	-	-	-	31	0.78**	9.12
10	233	149	24	24	36	5	1	-	8	3	-	2	2	2	2	25	0.63	10.73*
20	352	311	12	10	19	14	1	2	7	7	-	-	-	-	-	31	0.78**	8.81
40	186	123	18	22	23	-	1	1	7	-	-	-	3	-	-	12	0.30	6.45
80	194	156	17	5	16	8	-	-	3	-	-	-	-	-	-	11	0.28	5.67
Total	1305	1006	89	88	122	39	4	3	33	15	0	7	5	2	2	110	2.77	40.78
% Freq		77.09	6.82	6.74	9.35	35.45	3.64	2.73	30.00	13.64	0.00	6.36	4.54	1.82	1.82	100		

NC- Negative control; PC – Positive control; % Freq – % frequency observed in test experiment; Total: sum values in test experiments

*, **Values significant respectively at $p < 0.05$ and $p < 0.01$

Table 4.4. Mitotic indices of *A. cepa* grown in CuONPs following 24 h exposure and 24 h recovery periods

Conc. (mg/L)	Effects of 24 h exposure			Effect of 24 h recovery		
	Number of dividing cells	Mitotic index	Mitotic inhibition	Number of dividing cells	Mitotic index	Mitotic inhibition
NC	188	47.00		158	39.50	
PC	131	32.80	30.32	160	40.00	-1.27
5	177	44.30	5.85	85	21.30	46.20
10	38	9.50**	79.79	44	11.00	72.15
20	0	0.00***	100.00	177	44.30	-12.03
40	0	0.00***	100.00	128	32.00	18.99
80	11	2.80***	94.15	104	26.00	34.18

Conc: Concentration; NC: Negative control (Distilled water)

PC: Positive control 10 ppm Pb(NO₃)₂. Lead nitrate

** , ***Values significant at p < 0.01 and p < 0.001 respectively

Number of cells scored per concentration = 4000

A decrease in MI was also observed in the *A. cepa* root cells exposed to 1:1 mixture of both NPs for 24 h except at 5 and 10 mgL⁻¹ (Table 4.6). The observed MI were 72.00, 63.50, 19.50, 30.80 and 17.30 at 5, 10, 20, 40 and 80 mgL⁻¹ of the 1:1 mixture respectively. There was a concentration dependent decrease in MI (83.80, 63.80, 56.00, 38.30, and 11.5 %) of the root cells in the recovery solution at tested concentrations. However, there was a decrease at the two (2) highest concentrations and an increase at the 3 lowest concentration compared to the negative control. The percentage frequency of CA in dividing cells (3.82, 9.45, 5.13, 13.82 and 11.60 %) increased following exposure to the NPs 1:1 mixture in comparison with the negative control. Similarly, the frequency of dividing cells increased during the recovery period except at 5 mgL⁻¹. The total frequency of CA induced by the NPs 1:1 mixture in the dividing cells, however, decreased in the recovery solution from 43.82 % to 34.87.

The normal chromosome and different stages of cell division (Figure 4.8) observed were interphase, prophase, metaphase and anaphase and telophase. Chromosome aberrations observed include sticky chromosome at different mitotic stages (Figure 4.9), spindle disturbance (Figure 4.10), disturbed metaphase (Figure 4.11), binucleated cells and polar deviations (Figure 4.12), chromosome non disjunction (Figure 4.13), micronucleated cells (Figure 4.14), anaphase bridges and C – mitosis (Figures 4.15 and 4.16 respectively). The frequency of the different chromosome aberrations observed are presented on Tables 4.3, 4.5 and 4.7. Notably, sticky chromosomes were the most frequent CA observed in all the exposed and recovery *A. cepa* cells. This is followed by spindle disturbance and other aberrations associated with spindle dysfunction. Other predominant aberrations in the AgNPs groups are nuclear abnormalities (micronucleated cells, binucleated cells), c – mitosis and distributed metaphase. Also, in the CuONPs and the NPs 1:1 mixture group, the nuclear abnormalities (micronucleated cells, binucleated cells) and pole deviations, as well as distributed metaphase, were also observed. A two-way ANOVA comparison of the effect of 24 h recovery period on *A. cepa* cells following exposure to the NPs and their 1:1 mixture revealed significant increase ($p < 0.05$) only at 20 mgL⁻¹ in the CuONPs and 1:1 mixture groups (Figure 4.4). However, generally, there was a decrease in MI observed at 72 h exposure period following recovery period (at 96 h) in the AgNPs

exposed root cells while an increase was observed in the root cells CuONPs and the 1:1 mixture.

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Table 4.5. cytological effects of CuONPs on *A. cepa* root tips following 24 h exposure and 24 h recovery periods

Conc. (mg/L)	Mitosis					Chromosome aberrations									Percentage frequency of aberrant cells based on:	
	No. of dividing cells	No of cells at prophase	No of cells at metaphase	No of cells at anaphase	No of cells at telophase	Sticky chromosome	Anaphase bridge	Distributed metaphase	Spindle disturbance	C – mitosis	Vagrant chromosome	Pole deviation	Binucleated	Total aberrant cells	Total cells scored	No. of dividing cells
24 h exposure period																
NC	188	140	17	15	16	1	-	4	-	-	-	-	-	5	0.13	2.66
PC	131	94	12	23	2	24	-	1	5	-	-	1	31	0.78**	23.66**	
5	177	136	9	14	18	8	-	1	-	-	1	2	12	0.30	6.78	
10	38	36	-	1	1	2	-	-	-	-	-	-	2	0.05	5.26	
20	-	-	-	-	-	-	-	-	-	-	-	-	-	0.00	0.00	
40	-	-	-	-	-	-	-	-	-	-	-	-	-	0.00	0.00	
80	11	11	-	-	-	-	-	-	-	-	-	-	-	0.00	0.00	
Total	226	183	9	15	19	10	0	1	0	0	0	1	2	14	0.35	12.04
% Freq		80.97	3.98	6.64	8.41	71.43	0.00	7.14	0.00	0.00	0.00	7.14	14.29	100		
24 h recovery period																
NC	158	142	5	8	3	8	-	-	-	1	-	-	9	0.23	5.70	
PC	160	102	23	24	11	12	1	1	5	5	-	-	24	0.60*	15.00**	
5	85	75	6	1	3	4	-	1	-	-	1	-	6	0.15	7.06*	
10	44	35	6	2	1	1	-	-	-	-	-	-	1	0.03	2.27*	
20	177	156	7	8	6	2	-	-	-	-	-	-	2	0.05	1.13*	
40	128	118	8	-	2	2	-	-	-	-	-	1	3	0.08	2.34*	
80	104	104	-	-	-	-	-	-	-	-	-	-	-	0.00	0.00*	
Total	538	488	27	11	12	9	-	1	-	-	1.00	-	1.00	12	0.31	12.8
% Freq		90.71	5.02	2.04	2.23	75.1	0.00	8.33	0.00	0.00	8.33	0.00	8.33	100		

NC- Negative control; PC – Positive control; % Freq – % frequency observed in test experiments; Total: sum of values in test experiments

*Values significant at $p < 0.05$, ** $p < 0.01$

Table 4.6. Mitotic indices of *A. cepa* grown in 1:1 mixture of AgNPs and CuONPs following 24 h exposure and 24 h recovery periods

Conc. (mg/L)	Effects of 24 h exposure			Effect of 24 h recovery		
	Number of dividing cells	Mitotic index	Mitotic inhibition	Number of dividing cells	Mitotic index	Mitotic inhibition
NC	188	47.00		158	39.50	
PC	131	32.80	30.32	160	40.00	-1.27
5	288	72.00	-53.19	335	83.80*	-112.03
10	254	63.50	-35.11	255	63.80	-61.39
20	78	19.50	58.51	224	56.00	-41.77
40	123	30.80	34.57	153	38.30	3.16
80	69	17.30	63.3	46	11.50	70.89

Conc: Concentration; NC: Negative control (Distilled water)

PC: Positive control 10 ppm Pb(NO₃)₂. Lead nitrate

*Values significant at p <0.05

Number of cells scored per concentration = 4000

Table 4.7. Cytological effects of 1:1 mixture of AgNPs and CuONPs on *A. cepa* root tips following 24 h exposure and 24 h recovery periods

Conc. (mg/L)	Mitosis					Chromosome aberrations										Frequency (%) of aberrant cells based on:	
	No. of dividing cells	No of cells at prophase	No of cells at metaphase	No of cells at anaphase	No of cells at telophase	Sticky chromosome	Anaphase bridge	Distributed metaphase	Spindle disturbance	C – mitosis	Vagrant chromosome	Pole deviation	Binucleated Cells	Chromosome non-disjunction	Total aberrant cells	Total cells scored	No. of dividing cells
24 h exposure period																	
NC	188	140	17	15	16	1	-	4	-	-	-	-	-	-	5	0.13	2.66
PC	131	94	12	23	2	24	-	1	5	-	-	1	-	31	0.78	23.66**	
5	288	236	22	10	20	4	-	2	3	-	-	1	-	11	0.28	3.82	
10	254	172	33	23	26	9	4	2	6	-	-	2	1	24	0.60*	9.45	
20	78	67	5	3	3	4	-	-	-	-	-	-	-	4	0.10	5.13	
40	123	74	20	11	18	11	-	2	3	1	-	-	-	17	0.43	13.82	
80	69	47	9	6	7	2	-	2	-	1	-	1	2	8	0.2	11.60	
Total	743	549	80	47	67	30	4	8	12	2	0	4	3	64	1.61	43.82	
% Freq						46.88	6.25	12.50	18.75	3.13	0.00	6.25	4.69	100			
24 h recovery period																	
NC	158	142	5	8	3	8	-	-	-	1	-	-	-	9	0.23	5.70	
PC	160	102	23	24	11	12	1	1	5	5	-	-	-	24	0.60	15.00**	
5	335	245	35	20	35	3	2	2	6	-	2	-	-	15	0.38**	4.48	
10	255	166	31	18	40	6	1	3	6	-	-	1	-	17	0.43	6.67	
20	224	138	29	26	31	8	4	2	4	-	-	1	-	19	0.48	8.48	
40	153	86	25	19	23	6	-	1	3	-	-	-	-	10	0.25	6.54	
80	46	38	4	3	1	3	-	-	1	-	-	-	-	4	0.10	8.70	
Total	1013	673	124	86	130	26	7	8	20	0	2	2	0.00	65	1.64	34.87	
% Freq						40.00	10.77	12.31	30.77	0.00	3.08	3.08	0.00	100			

NC- Negative control; PC – Positive control; % Freq – % frequency observed in test experiment; Total: sum of values in test experiments.

*Values significant at $p < 0.05$, ** $p < 0.01$

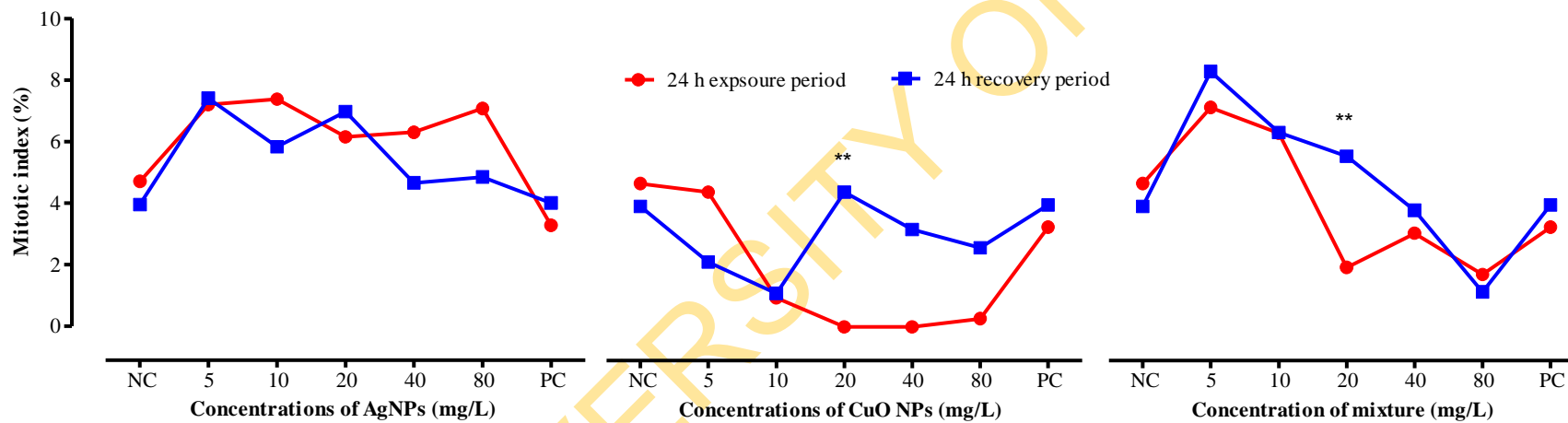


Figure 4.6. Comparative mitotic indices of *A. cepa* root cells after 24 h exposure to AgNPs, CuONPs, and their 1;1 mixture, and a 24 h recovery period

Using two-way ANOVA **Values significant at $P < 0.01$ between 24h treatment and 24 recovery periods. NC- Negative control (Distilled water); PC - Positive control [$\text{Pb}(\text{NO}_3)_2$: Lead nitrate]

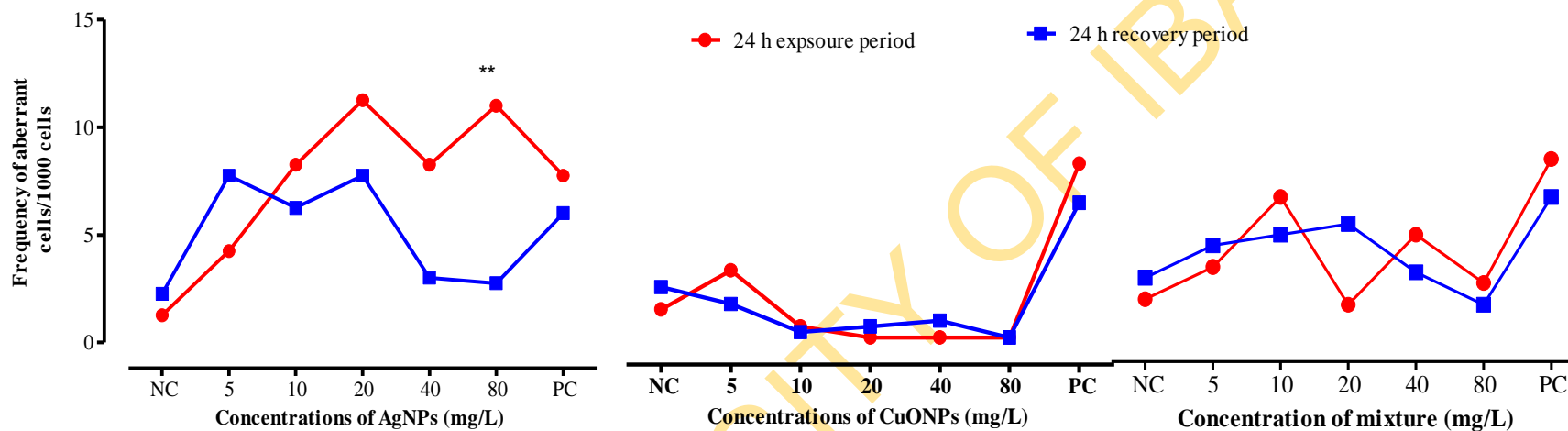


Figure 4.7. Variations in frequency of chromosome aberration induced *A. cepa* root cells after 24 h exposure to AgNPs, CuONPs, and their 1:1 mixture, and a 24 h recovery period

Using a two-way ANOVA **Values is significant at $p < 0.01$ between 24 h exposure and 24 h recovery periods.
 NC- Negative control (Distilled water); PC - Positive control [$\text{Pb}(\text{NO}_3)_2$: Lead nitrate]

Table 4.8. Interactive effects of 1:1 mixture of AgNPs and CuONPs on the frequency of chromosome aberrations in *Allium cepa* root tips

Concentrations (mg/L)	Interaction factor of CA (IF ± SE _{IF})	
	72hrs	96hrs
NC	-	-
5	-0.32 ± 0.22	-0.32 ± 0.50
10	-0.15 ± 0.34	0.00 ± 0.16
20	-0.90 ± 0.35	-0.12 ± 0.35
40	-0.27 ± 0.39	0.10 ± 0.18
80	-0.77 ± 1.10	0.05 ± 0.15

Negative IF value = antagonistic interaction; positive IF = synergistic interaction

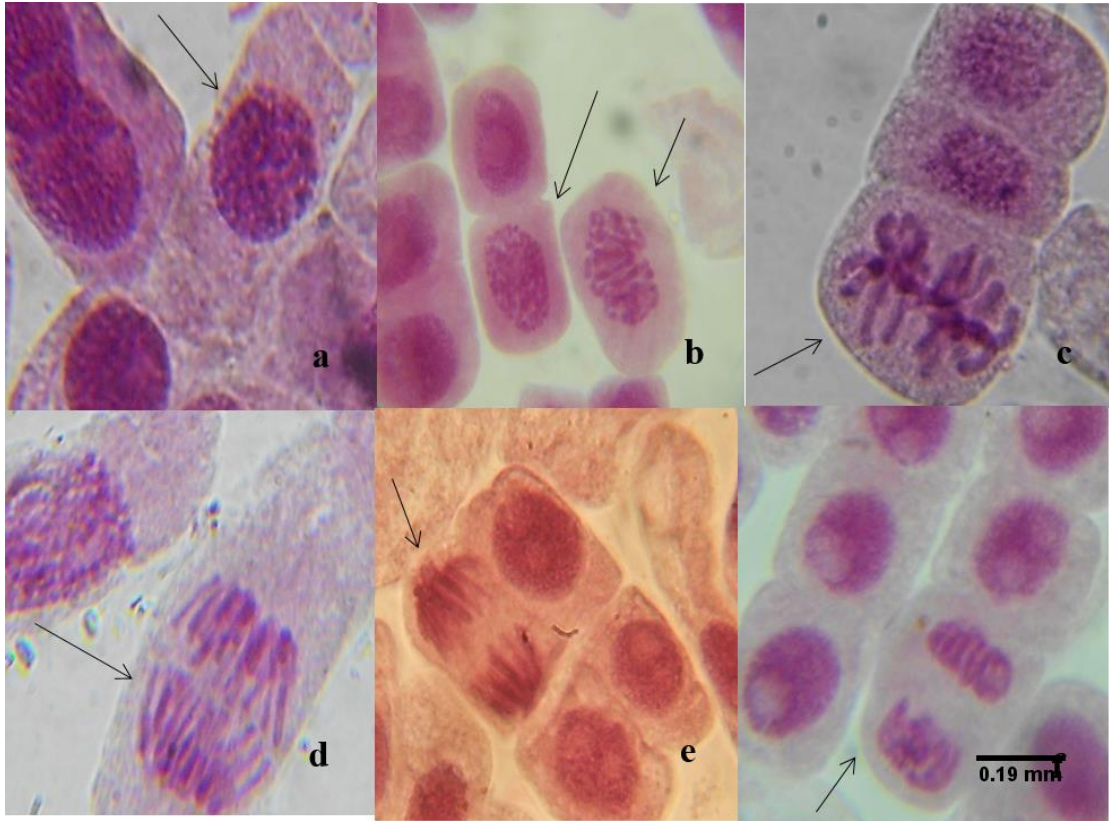


Figure 4.8. Normal mitotic stages observed in *Allium cepa* root tips: (a) interphase (b) prophase (c) metaphase (d - e) anaphase (f) telophase (x 1000)

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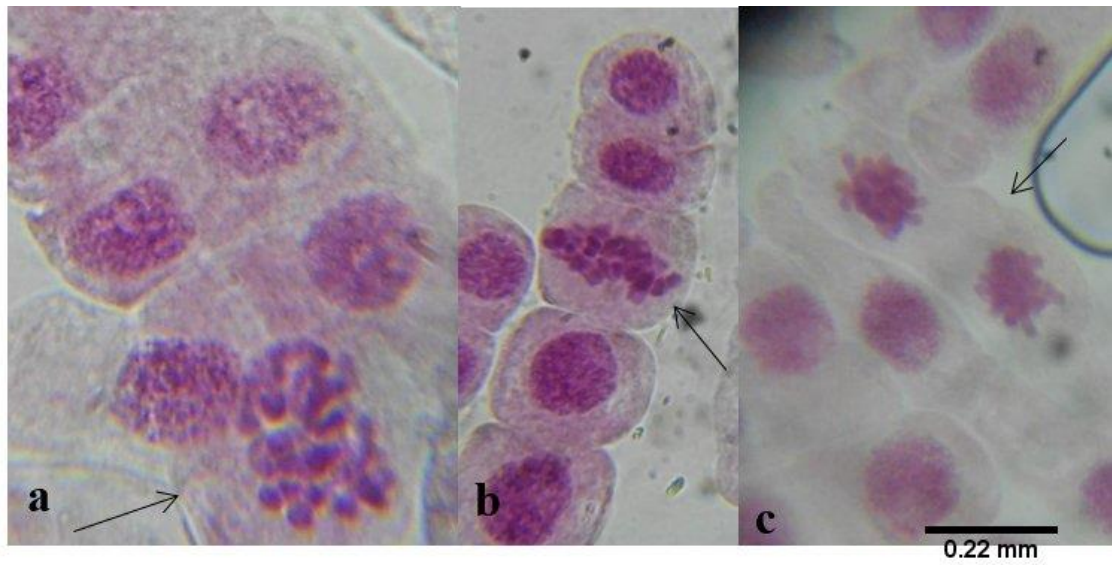


Figure 4.9. Sticky chromosomes in *Allium cepa* root tips exposed to AgNPs, CuONPs and their 1:1 mixture. Sticky chromosomes at: (a) prophase (b) metaphase (c) anaphase (x 1000)

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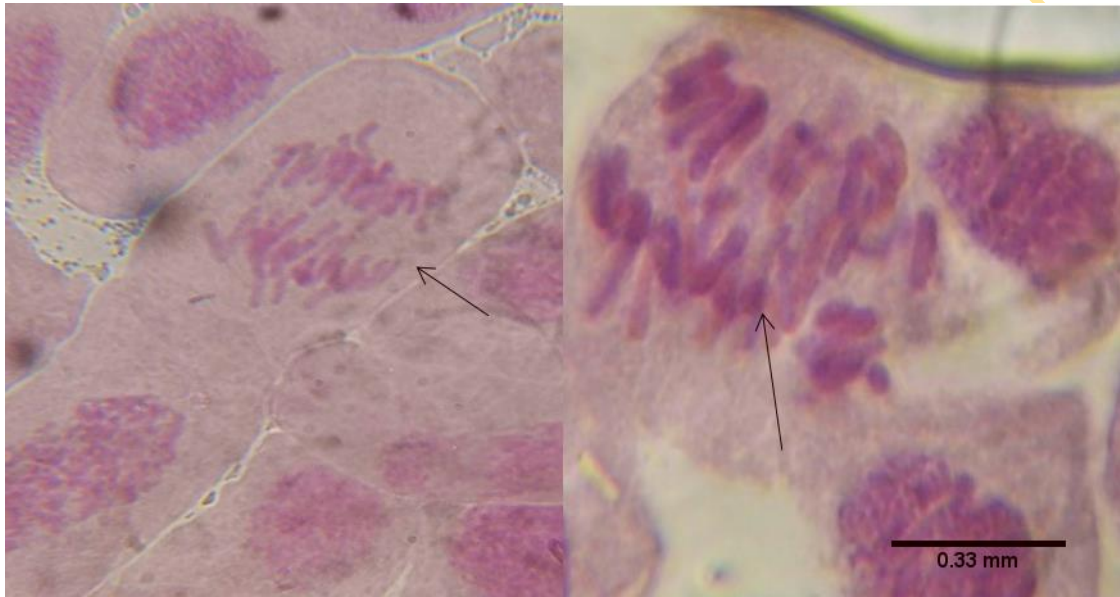


Figure 4.10. Spindle disturbances observed in *Allium cepa* root tips exposed to AgNPs and the 1:1 mixture (x 1000)

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Figure 4.11. Distributed metaphase induced in *Allium cepa* root tips exposed to AgNPs (x 1000)

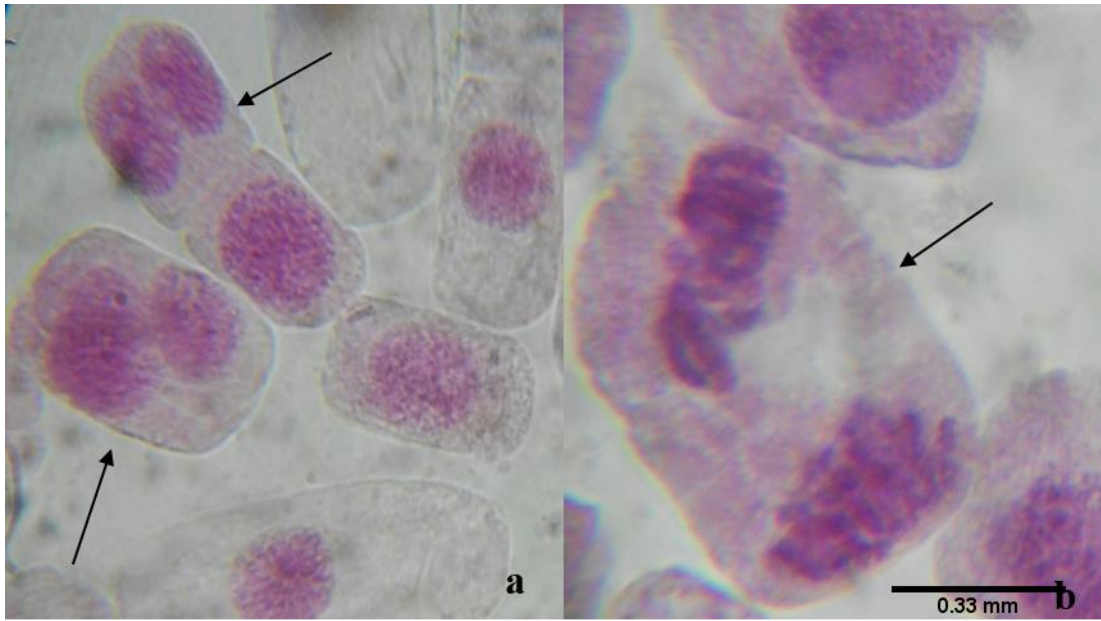


Figure 4.12. Binucleated cells (a) and Polar deviation (b) induced in *Allium cepa* root tips exposed to AgNPs, CuONPs and their 1:1 mixture (x 1000)

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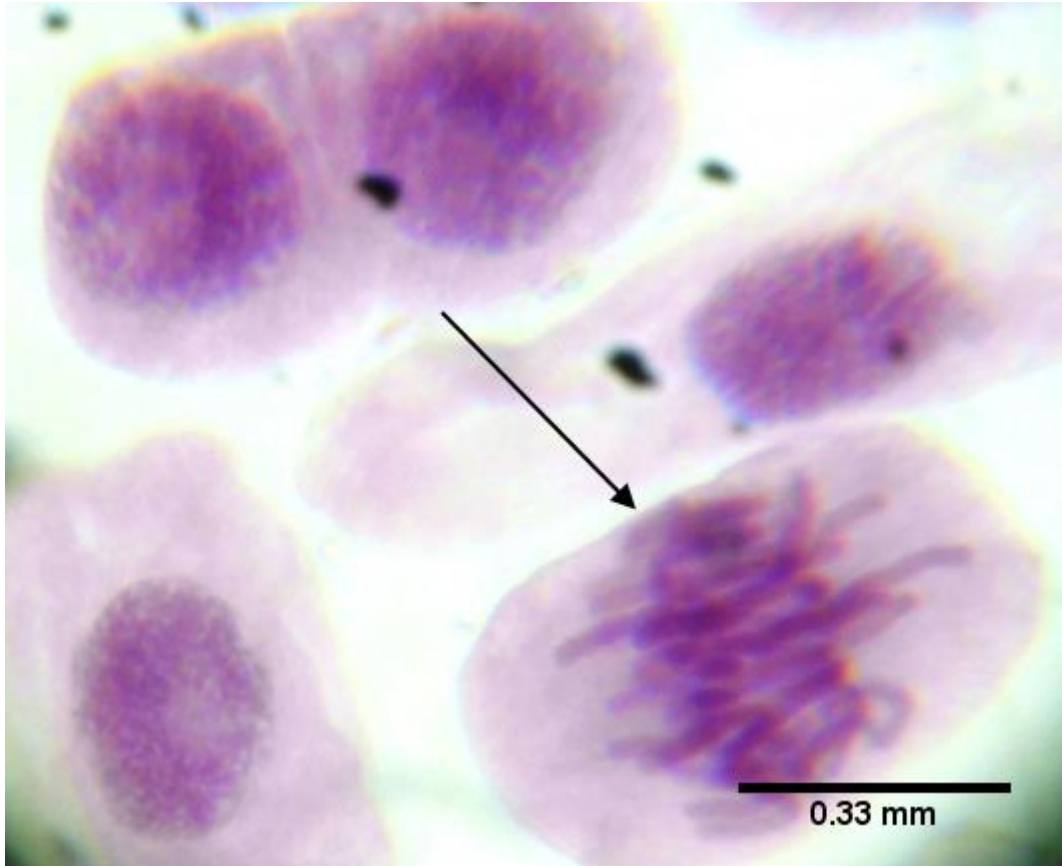


Figure 4.13. Chromosome non – disjunction at anaphase in *Allium cepa* roots exposed to 1:1 mixture of AgNPs and CuONPs (x 1000)

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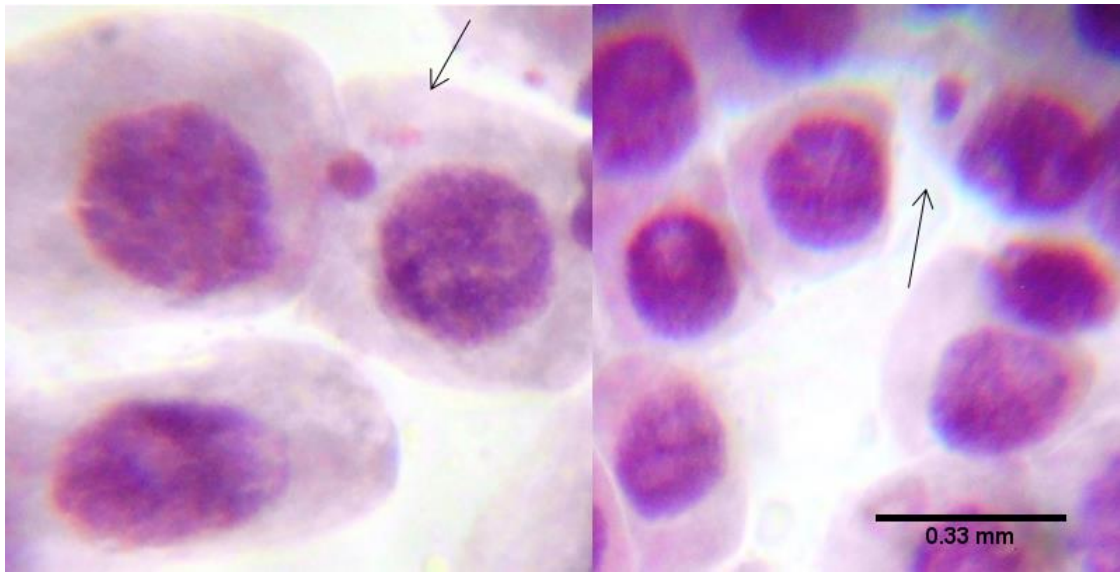


Figure 4.14. Micronucleated cells induced in *Allium cepa* root tips exposed to AgNPs, CuONPs and their 1:1 mixture (x 1000)

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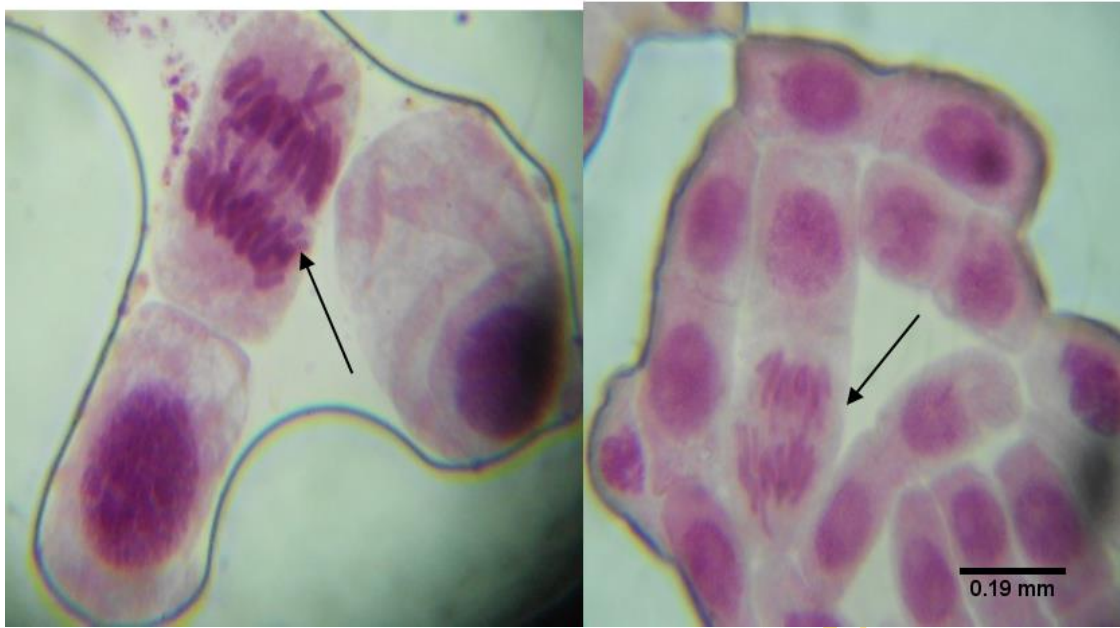


Figure 4.15. Anaphase bridges induced in *Allium cepa* root tips exposed to AgNPs, CuONPs and their 1:1mixture (x 1000)

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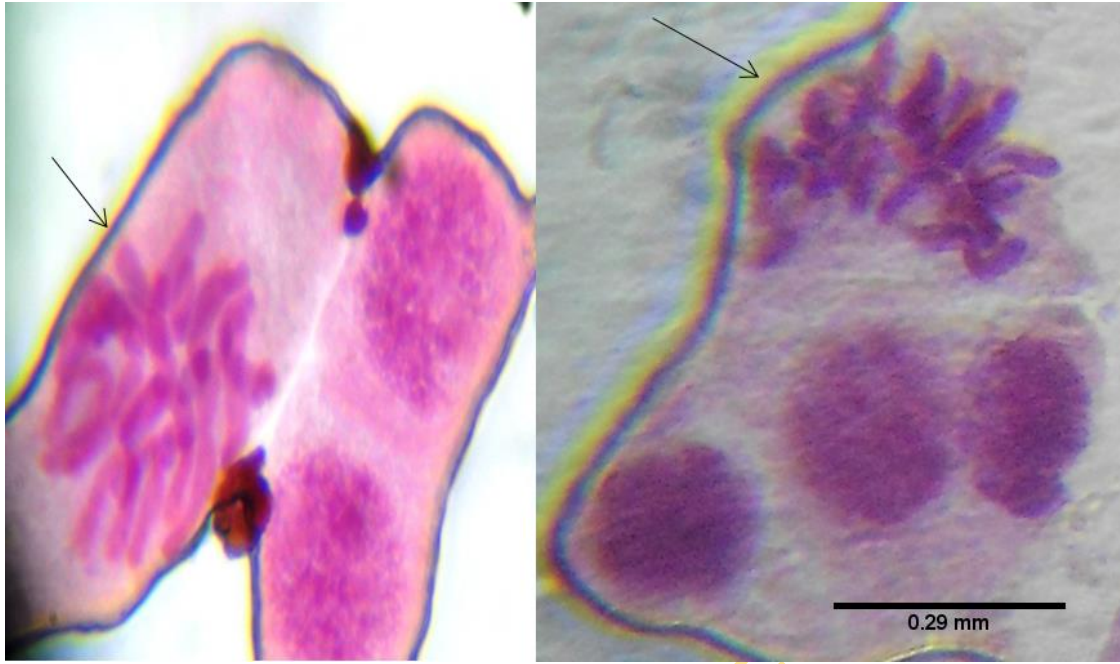


Figure 4.16. C – mitosis induced in *Allium cepa* root tips exposed to AgNPs and the 1:1 mixture (x 1000)

For the effect of recovery period on the frequency of observed aberrations, significant ($p < 0.05$) recovery was observed only at 80 mgL^{-1} in the AgNPs group. However, the frequency of CA in the AgNPs group generally decreased while it was generally higher in the CuONPs and 1:1 mixture group (Figure 4.5). The interaction factor (IF) analysis of the 1:1 mixture of the nanoparticle revealed an antagonistic interaction (Table 4.8) which is indicated by negative IF values.

4.3 Cytogenotoxicity and systemic toxicity induced by silver and copper(II) oxide nanoparticles, and their 1:1 mixture in *C. gariepinus*

4.3.1 Acute toxicity of silver and copper(II) oxide nanoparticles in *C. gariepinus*

Range finding tests carried out using *Clarias gariepinus* showed that both copper(II) oxide (CuO) and silver (Ag) nanoparticles NPs have low lethal toxicity in *C. gariepinus*. The LC_{50} was considered indeterminate following exposure for 96 h to CuONPs (100mg/L and 500mg/L) and AgNPs (10mg/L and 100mg/L) which resulted in percentage mortalities of 15% and 10% respectively. The LC_{50} was considered to be above 100 mg/L which is the threshold concentration according to OECD 203 on fish acute toxicity testing

4.3.2 Micronuclei and other nuclear abnormalities induced by AgNPs, CuONPs and their 1:1 mixture in *C. gariepinus*

Figures 4.17 – 4.19 shows the frequency of MN induced in the peripheral blood erythrocytes of juvenile *C. gariepinus* exposed to 6.25- 100 mg/L concentrations of each of the NPs and their 1:1 mixture for 7 and 28 days respectively. AgNPs significantly ($p < 0.05$) induced MN at both exposure periods (Figure 4.17). The highest frequency of MN was observed at 50 mg/L at both 7 (12.45 ± 1.77) and 28 (7.73 ± 0.86) days respectively. The frequencies of MN observed decreased with increase in exposure period but were significantly ($p < 0.05$) different at 25 mg/L and 50 mg/L concentrations.

Similarly, CuONPs significantly ($p < 0.05$) induced MN at 12.5 – 100 mg/L following 7 and 28 –day exposure periods (Figure 4.18). There was a decrease in frequency of MN as the exposure period increased but were only significant ($p < 0.05$) at 25 and 50

mg/L. The NPs 1:1 mixture induced MN at both exposure periods with the highest frequency

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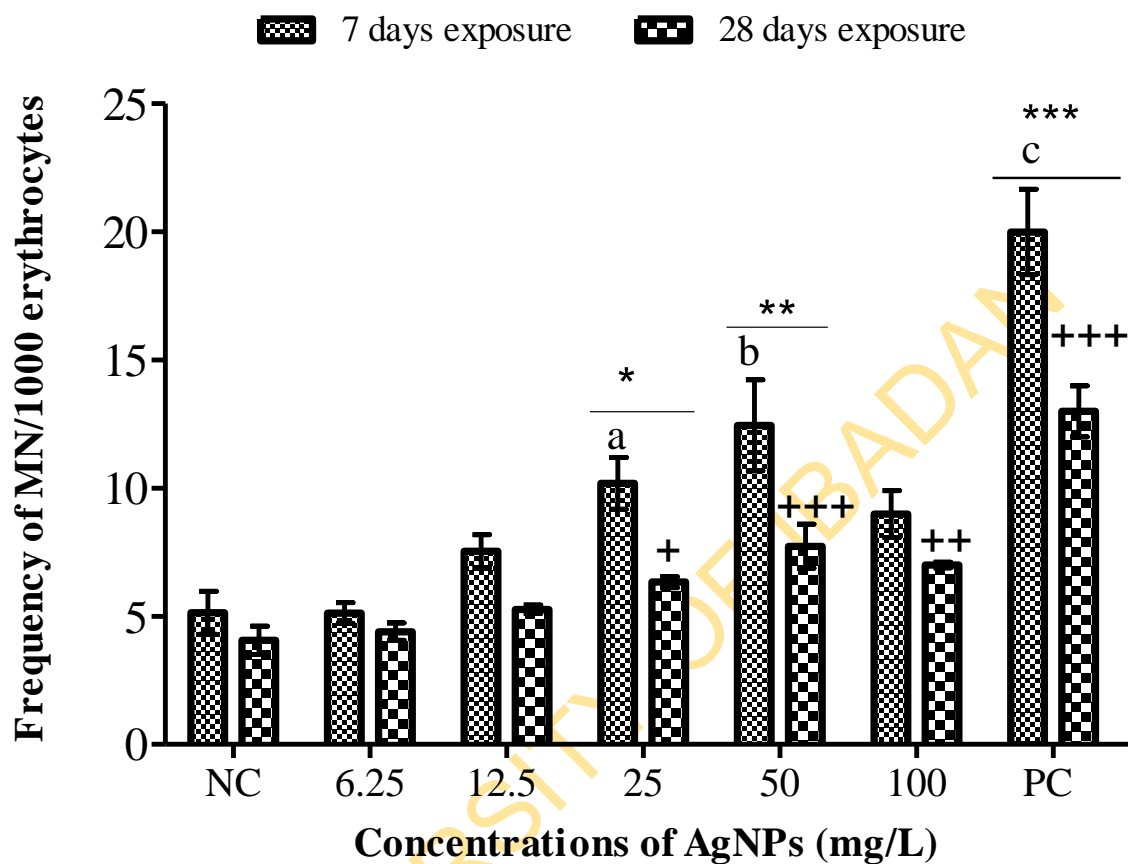


Figure 4.17. 1 Frequencies of micronuclei induced in peripheral erythrocytes of juvenile *Clarias gariepinus* exposed to different concentrations of silver nanoparticles (Ag NPs) for 7 and 28 days

NC- Negative control (dechlorinated tap water); PC - Positive control (0.05 mL/L Benzene)

Following a one-way analysis of variance (ANOVA), ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ at 7 days and ⁺ $p < 0.05$, ⁺⁺ $p < 0.01$, ⁺⁺⁺ $p < 0.001$ at 28 days compared with corresponding control. Using two-way ANOVA ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ between 7- and 28-days exposure periods.

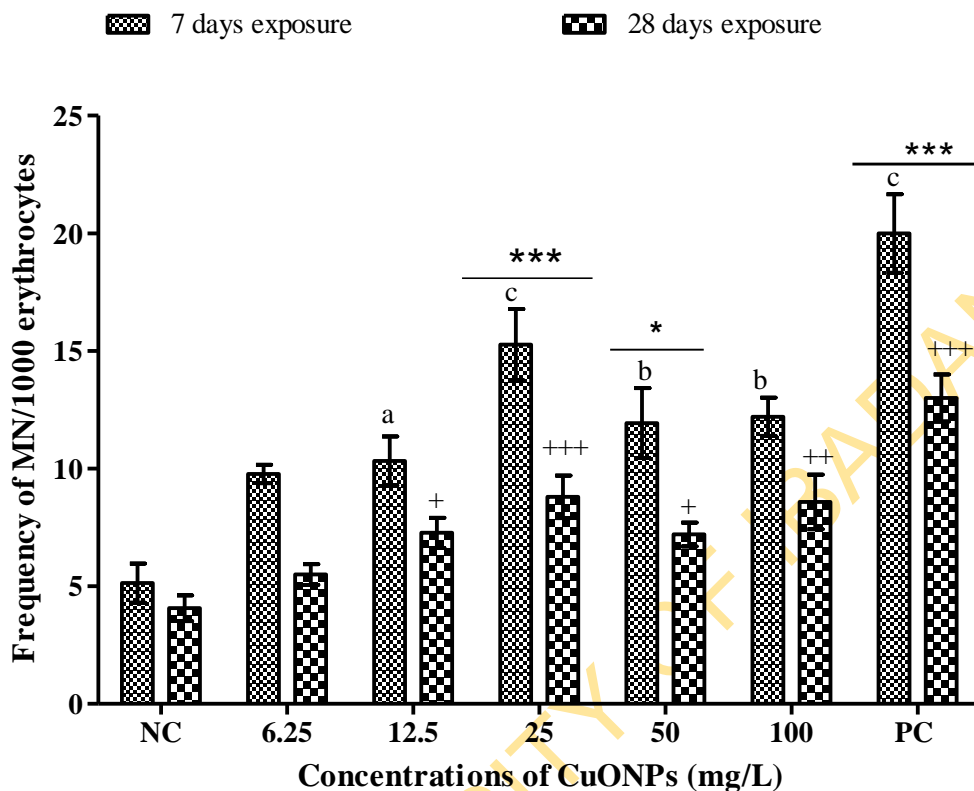


Figure 4.18. Frequencies of micronuclei induced in peripheral erythrocytes of juvenile *Clarias gariepinus* exposed to different concentrations of copper(II) oxide nanoparticles (CuONPs) for 7 and 28 days

NC- Negative control (dechlorinated tap water); PC - Positive control (0.05 mL/L Benzene)

Following a one-way analysis of variance (ANOVA), ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ at 7 days and ⁺ $p < 0.05$, ⁺⁺ $p < 0.01$, ⁺⁺⁺ $p < 0.001$ at 28 days compared with corresponding control. Using two-way ANOVA ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ between 7 and 28 day exposure periods.

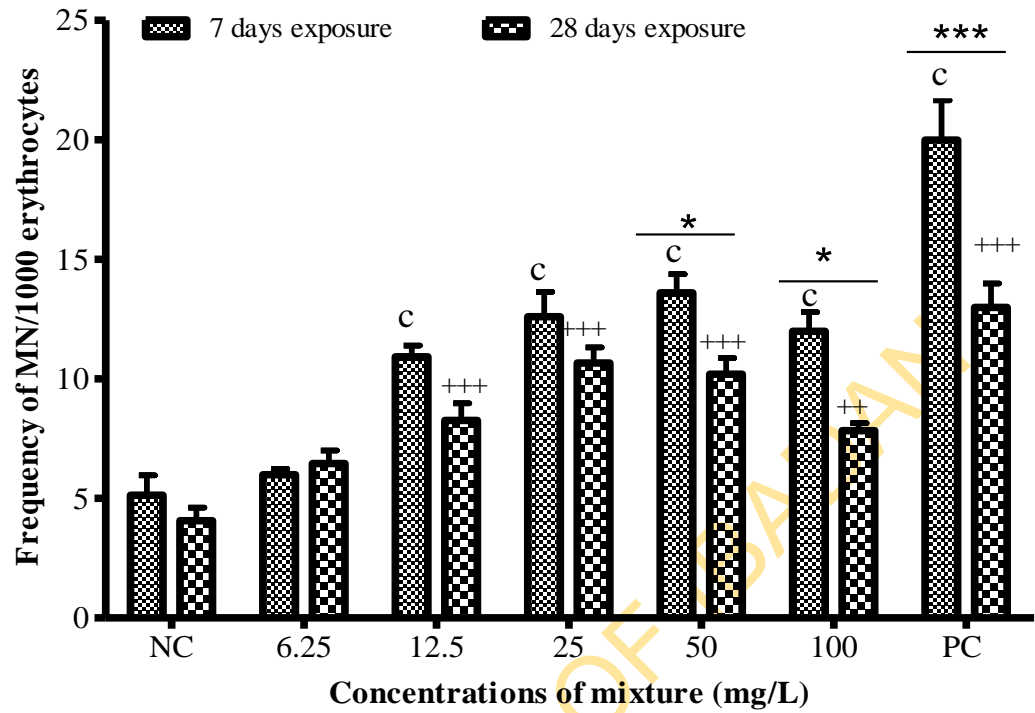


Figure 4.19. Frequencies of micronuclei induced in peripheral erythrocytes of juvenile *Clarias gariepinus* exposed to different concentrations of 1:1 mixture of AgNPs and CuONPs for 7 and 28 days.

NC- Negative control (dechlorinated tap water); PC - Positive control (0.05 mL/L Benzene)

Following a one-way analysis of variance (ANOVA), ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ at 7 days and, ⁺⁺ $p < 0.01$, ⁺⁺⁺ $p < 0.001$ at 28 days compared with corresponding control. Using two-way ANOVA ^{*} $p < 0.05$, ^{***} $p < 0.001$ between 7- and 28-days exposure periods.

Table 4.9. Effects of Ag NP, CuO NPs and their 1:1mixture on the frequency of total nuclear abnormalities in peripheral erythrocytes of juvenile *Clarias gariepinus* after 7- and 28-days exposure period

Concentrations (mgL ⁻¹)	Frequency of TNA per 1000 erythrocyte		
	Ag NP	CuO NP	Ag + CuO NPs
7 days exposure			
NC	0.07 ± 0.07	0.07 ± 0.07	0.07 ± 0.07
6.25	0.20 ± 0.13	0.33 ± 0.19	2.33 ± 1.43
12.5	0.60 ± 0.37	0.27 ± 0.19	7.33 ± 2.1*1
25	0.13 ± 0.08	1.13 ± 0.82	1.07 ± 0.43
50	0.53 ± 0.37	0.67 ± 0.52	1.33 ± 0.55
100	0.13 ± 0.91	0.66 ± 0.66	3.17 ± 2.64
PC	4.70 ± 2.60 *	4.70 ± 2.60*	4.70 ± 2.60*
28 days exposure			
NC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
6.25	0.53 ± 0.53	0.07 ± 0.07	3.47 ± 1.42
12.5	1.67 ± 1.29	2.20 ± 0.90	4.20 ± 0.71
25	0.00 ± 0.00	0.13 ± 0.13	0.00 ± 0.00
50	0.00 ± 0.00	3.53 ± 0.58	1.34 ± 1.34
100	0.00 ± 0.00	4.08 ± 2.20	0.00 ± 0.00
PC	6.00 ± 3.38*	6.00 ± 3.38*	6.00 ± 3.38*

NC- Negative control (dechlorinated tap water); PC - Positive control (0.05 mL/L Benzene), values are mean ± SE, * values are significant at p < 0.05

of MN observed at 50 and 25 mg/L concentrations at 7 and 28 days respectively. However, marked decreases in frequency of MN between 7- and 28-days exposure periods were observed only at 50 and 100 mg/L (Figure 4.19).

Table 4.9 shows the frequencies of total nuclear abnormalities (TNA) observed alongside the MN in the peripheral blood erythrocytes of the NPs exposed fish. Though statistically insignificant ($p > 0.005$), the observed frequencies were high in comparison to the negative control group on the 7th day. The frequency of the TNA, however, decreased following longer exposure time at 28 days. The NPs 1:1 mixture induced highest frequencies of TNA in peripheral blood of *C. gariepinus* at both 7 and 28 days. The frequencies of TNA induced following exposure to CuONPs were higher than those observed in the AgNPs group. The highest frequencies of TNA were observed in the 12.5mg/L mixture(1:1) group at both exposure periods (7.33 ± 2.11 and 4.20 ± 0.71).

Figures 4.20 – 4.21 shows the micronucleated erythrocytes and nuclear abnormalities in the peripheral erythrocytes of *C. gariepinus*. The observed nuclear abnormalities include binucleated cells, notched and blebbed nuclei. The interaction factor analysis of the effect of the 1:1 mixture of both silver and copper(II) oxide nanoparticles (Table 4.10) shows an antagonistic interaction (Negative IF value) of AgNPs and CuONPs on MN induction at both exposure periods with the frequency of MN induced by the 1:1 mixture being higher than the frequency induced by individual NPs but not more than the sum of the MN frequency of both nanoparticles. Interaction factor values at 7 days were -2.54 ± 0.61 , -1.79 ± 1.31 , -7.72 ± 2.09 , -4.13 ± 2.17 and -4.06 ± 1.57 at 6.25, 12.25, 25.00, 50.00 and 100.00 mg/L⁻¹ respectively. The interaction effects of the NPs were lower at 28 days exposure (-0.2 ± 0.97 , -0.40 ± 1.13 , 0.67 ± 1.20 and -3.68 ± 1.20 at 12.5, 25.00, 50.00 and 100.00 mg/L).

4.3.3 Haematological changes in *C. gariepinus*

Haematological parameters of fish exposed to AgNPs, CuONPs and their 1:1 mixture are presented in Tables 4.11 – 4.15. *C. gariepinus* exposure to AgNPs resulted in decreased PCV, Hb, RBC and MCHC (Table 4.11) while MCH and MCV increased compared to the control. The decrease in RBC, Hb, and PCV were however only significant at the 28th day exposure period. AgNPs also significantly induced decreases ($p < 0.05$) in WBC,

heterophils count (leucopenia) and platelet at the 28 day exposure period compared to the negative control (Table 4.11).

CuONPs caused a decrease in PCV, Hb, RBC, and MCHC with a concomitant increase in MCV and MCH values (Tables 4.12 and 4.13). Also, a significant decrease ($p < 0.05$) were also recorded in WBC, platelets and neutrophil counts at 28 days with a concomitant increase in lymphocytes count compared to the negative control values. The 1:1 mixture of the NPs induced decreases in PCV, Hb, RBC and MCV in peripheral erythrocyte of *C. gariepinus* at 7th and 28th days of exposure (Table 4.14)

4.3.4 Histopathological alterations in *C. gariepinus*

The histological changes observed in the skin, gills, liver and intestine of exposed fish are presented in figures 4.22 – 4.25. The epidermal skin layer of the negative control group comprises keratinocytes without visible lesions (Figure 4.22A) while those of the NPs-exposed fish presented with moderate hyperplasia of keratinocytes (Figure 4.22B), and alarm cells as well as presence of goblet cells (Figure 4.22C). The gills showed normal architecture in the negative control group with numerous and tall filaments, primary and secondary filament branching. However, the exposed fish showed moderate lamella hyperplasia and clumping and sloughing off of the secondary gill filament (Figure 4.23 B – D).

The hepatocytes of the unexposed *C. gariepinus* showed closely packed cells with large cytoplasm and centrally placed nucleus. The liver histology of the NPs – exposed fish revealed diffuse vacuolation of hepatocytes; diffuse vacuolar degeneration with foci of necrosis, centrilobular vacuolar degeneration and hepatic atrophies (Figure 4.24 B- D). The histological section of the intestines of exposed fish showed little or no alterations in the tissue structures. The intestinal structure of the negative control fish shows numerous tall closely packed villi. There were no visible lesions in the exposed fish except for moderate hyperplasia of cryptal cells.

4.3.5 Hepatic oxidative stress biomarkers in *C. gariepinus*

Table 4.15 shows the effect of subacute exposure of *C. gariepinus* to both silver and copper(II) oxide nanoparticles and their 1:1 mixture on the piscine antioxidant enzyme system. Both NPs and mixture altered the levels of the oxidative stress biomarkers. AgNPs significantly ($p < 0.05$) increased SOD activity compared with the control group and a concomitant increase in CAT activity. There was a significant increase in the lipid

peroxidation and concomitant increase in the activities of GSH, SOD and CAT of the antioxidant defense system. Furthermore, a significant ($p < 0.05$) decrease in MDA was elicited by significant depletion of CAT, SOD AND GSH molecules at the higher concentrations.

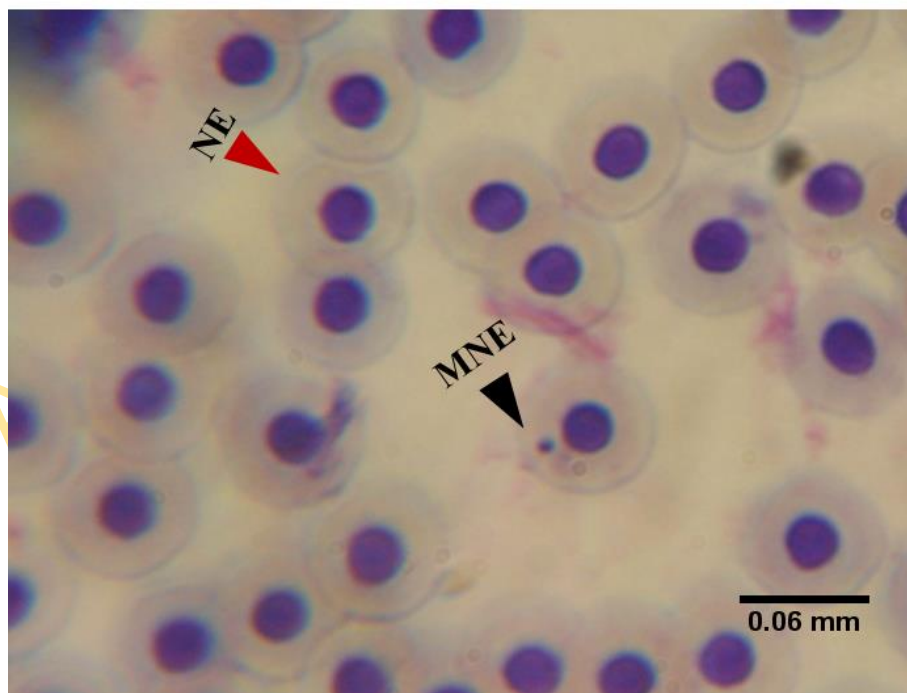
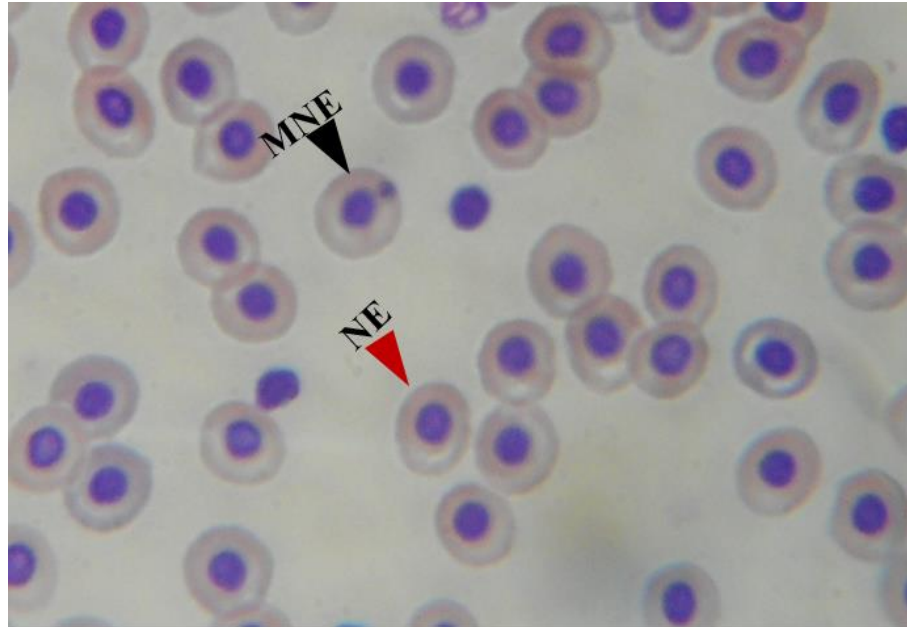


Figure 4.20. A and B Micronuclei (MN) induced in peripheral erythrocytes of *Clarias gariepinus* exposed to silver, copper(II) oxide nanoparticles and their 1:1

mixture; NE: Normal erythrocytes , MNE:micronucleated erythrocytes
(black arrow) (x 1000)

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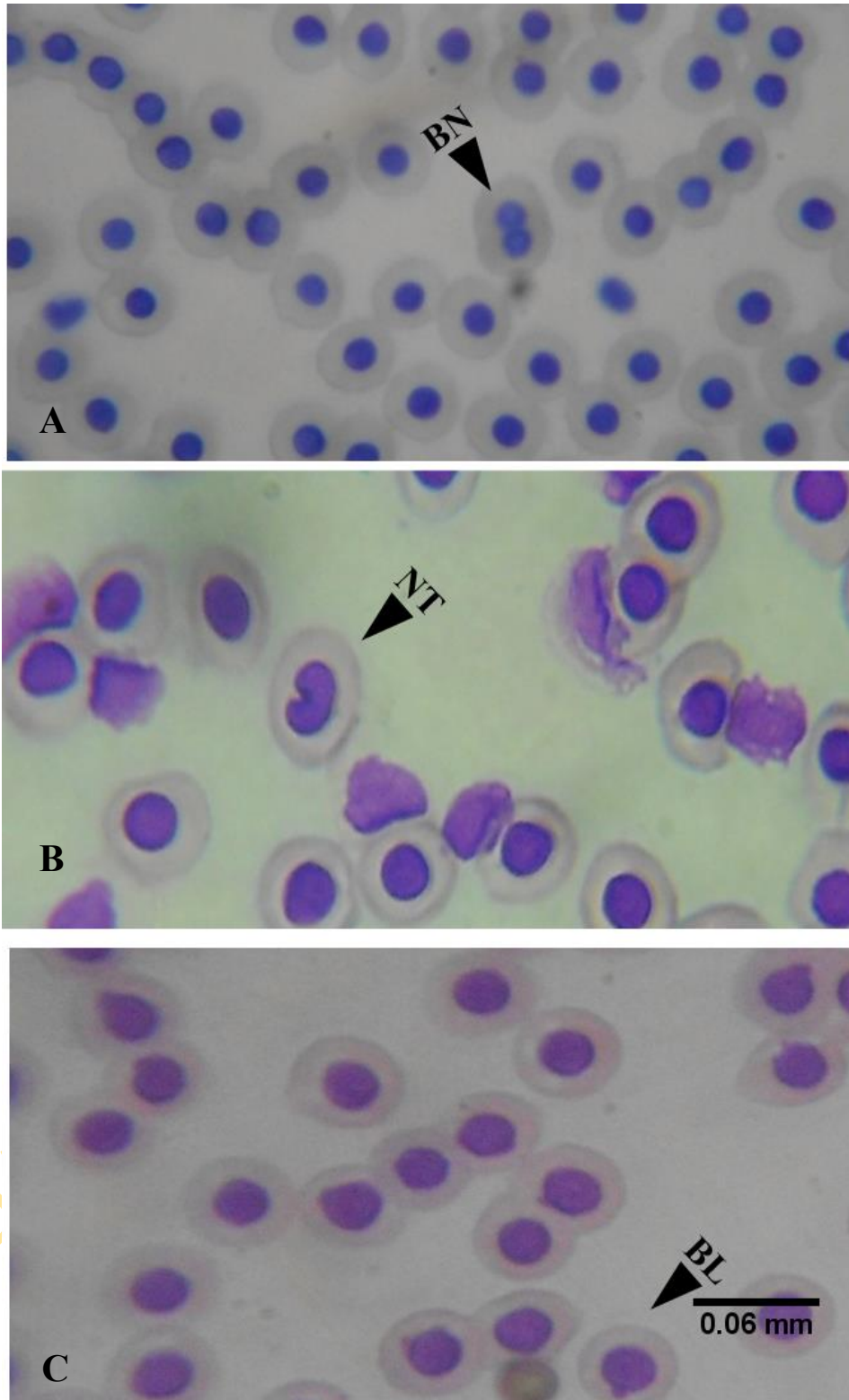


Figure 4.21. A, B and C: Nuclear abnormalities observed in peripheral erythrocytes of juvenile *C. gariepinus* exposed to silver, copper(II) oxide nanoparticles and their 1:1 mixture. (A) Binucleated (BN) cell (B) Notched nuclei (NT) (C) Blebbed nuclei (BL) (x 1000)

Table 4.10. Interactive effects of 1:1 mixture of AgNPs and CuONPs on Micronuclei induction in *Clarias gariepinus* following 7 and 28 days exposure periods

Concentrations (mg/L)	Interaction factor on MN (IF ± SE _{IF})	
	7 days	28 days
NC	-	-
6.25	-2.54 ± 0.61	0.63 ± 0.78
12.50	-1.79 ± 1.31	-0.2 ± 0.97
25.00	-7.72 ± 2.09	-0.40 ± 1.13
50.00	-4.13 ± 2.17	-0.67 ± 1.20
100.00	-4.06 ± 1.57	-3.68 ± 1.20

NB: IF = (AgNP+CuONPs - Control) - [(AgNPs - Control) + (CuONPs - Control)]
= (AgNP+CuONPs - AgNPs - CuONPs + Control)

Table 4.11. Red blood cell (RBC) and Red cell indices of juvenile *Clarias gariepinus* exposed to

Parameters	Concentrations (mg/L)	CuO NPs		Ag NPs	
		7 days	28 days	7 days	28 days
PCV	NC	34.00±6.44	35.80± 2.08	34.00±6.19	35.80 ± 2.08
	6.25	26.00±4.00	27.20± 1.88	29.80 ±1.92	38.80 ± 3.34
	12.5	29.00±8.31	24.40± 0.68*	26.60±6.65	32.60 ± 4.13*
	25	20.20±3.11*	28.20± 3.51	29.60±2.30	26.00 ± 3.11*
	50	26.40±2.88	21.20± 0.97*	26.67±12.66	22.60 ± 2.04
	100	24.00±9.38	28.20± 3.51	28.60±2.60	29.80 ± 2.03*
	PC	24.60±5.27	31.20± 3.61	24.60±5.27	31.20± 3.61
	Hb(g/dl)	NC	11.32±2.19	12.32± 0.57	11.32 ±2.19
6.25		8.47±1.37	8.88± 0.62*	9.72 ± 0.91	13.06 ± 1.06
12.5		9.50±2.63	8.34± 0.25*	8.72 ± 2.19	11.02 ± 1.44*
25		6.46±0.98*	9.44± 1.13*	9.48±1.07	8.44 ± 1.02*
50		8.76± 0.67	7.12± 0.27	8.73± 4.38	7.02 ± 0.71
100		7.85±3.07*	9.44± 1.13	9.16±0.83	9.72 ± 0.68*
PC		7.98±1.62*	10.34± 1.34	7.98±1.62	10.34 ± 1.34
RBC (x 10 ⁶)		NC	3.55±0.48	3.77± 0.10	3.55±0.48
	6.25	2.70±0.85	2.40± 0.47	3.41±0.08	3.92 ± 0.12
	12.5	2.88±0.67	2.19± 0.28	2.92±0.77	3.38 ± 0.27*
	25	1.71±0.47*	2.78± 0.52	3.56±0.21	2.03 ± 0.40*
	50	3.00±0.50	1.36± 0.04	2.22±1.57	2.15 ± 0.39
	100	2.12±0.84*	2.78± 0.52	3.19±0.48	3.20 ± 0.17
	PC	2.43±1.00	3.43± 0.35	2.43±1.00	3.43 ± 0.35
	MCHC (%)	NC	33.27±0.55	34.62± 1.52	33.27±0.55
6.25		32.54±0.84	32.65± 0.50	32.58±1.44	33.72 ± 0.20
12.5		32.86±1.07	34.17± 0.22	32.79±1.26	33.76 ± .261
25		32.00±0.92	33.58± 0.38	31.96±1.19	32.45 ± 0.36
50		33.30±1.8	33.64± 0.35	32.48±0.85	30.95 ± 0.47
100		32.70±0.78	33.58± 0.38	32.05±1.09	32.62 ± 0.39
PC		32.54±1.30	32.99± 0.72	32.54±1.30	32.99 ± 0.72
MCH(pg)		NC	31.84±3.37	32.67± 0.91*	31.84±3.37
	6.25	32.37±4.90	40.75± 4.75	28.51±2.69	33.16 ± 1.84
	12.5	33.20±5.02	40.56± 4.76	30.14±3.59	32.26 ± 1.93
	25	39.10±8.52	36.82± 4.11	26.71±3.41	44.46 ± 4.61
	50	29.57±2.88	52.53± 2.13	43.06±9.52*	34.67 ± 3.18
	100	37.34±2.69	36.82± 4.11	29.19±4.47	30.55 ± 2.13
	PC	35.34±7.69	30.26± 2.22	35.34±7.69	30.26 ± 2.22
	MCV(fl)	NC	95.66±9.66	94.78± 3.28	95.66±9.66
6.25		99.56±15.66	125.06± 14.96	87.39±5.25	98.47 ± 5.87
12.5		101.03±14.87	118.77±14.12	91.73±7.89	95.50 ± 5.42
25		122.17±26.07	109.18±11.05	83.38±8.03	136.74 ± 13.45
50		88.77±6.82	156.39±7.53	132.91±31.22*	112.40± 11.3
100		114.09±5.89	109.18±11.05	90.80±10.78	93.50 ± 5.84
PC		108.33±21.86	91.42±5.34	108.33±21.87	91.42 ± 5.34

NC- Negative control (Tap water) PC: Positive control 0.005 mL/L Benzene *Statistically significant (p< 0.05)

CuO NPs and Ag NPs for 7 and 28 days

Table 4.12. White blood cells counts of *Clarias gariepinus* exposed to CuONPs and AgNPs for 7 and 28 days

Parameters	Conn (mg/L)	CuO NPs		Ag NPs	
		7 days	28 days	7 days	28 days
WBC (x 10 ³)	NC	32.27±8.07	24.19±2.97	32.27±8.07	24.19 ± 2.97
	6.25	26.55±5.33	15.91±2.04	17.19±8.88	21.44 ± 1.48
	12.5	33.25±8.70	16.80±2.310	20.11±3.70	21.67 ± 1.34
	25	35.65±3.19	17.91±1.16*	13.93±3.33*	12.53 ± 1.27*
	50	36.71±3.52	19.43±3.35	33.15±1.79	15.85 ± 1.23*
	100	16.11±3.98*	17.91±1.16*	24.23±8.11	13.84 ± 1.16*
	PC	19.42±8.18*	24.55±1.11	19.42±8.18	24.55 ± 1.11
PLATELET S (x 10 ⁶)	NC	0.28 ±0.01	0.33±0.06	0.28±0.01	0.33 ± 0.06
	6.25	0.27± 0.01	0.21±0.03	0.75±0.01	0.24 ± 0.02
	12.5	0.21±0.01	0.30±0.03	0.22±0.08	0.28 ± 0.03
	25	0.29±0.08	0.16±0.03*	0.18±0.06	0.13 ± 0.01
	50	0.87±0.01	0.30±0.02	0.38±0.01	0.14 ± 0.02*
	100	0.19±0.04	0.16±0.03*	0.22±0.08	0.11 ± 0.08*
	PC	0.27±0.01	0.26±0.03	0.27±0.10	0.26 ±0.04
LYMPH (%)	NC	51.80±6.61	44.60±1.03	51.80±6.61	44.60 ± 1.02
	6.25	49.00±4.58	51.40±2.38	60.00±4.95	62.40 ± 3.56*
	12.5	59.00±7.87	57.00±3.46*	54.60±6.02	64.0000
	25	46.20±6.06	54.80±2.71*	64.60±4.82	69.00 ± 2.07
	50	46.00±6.82	57.00±2.77*	44.33±6.66	66.20 ± 2.96*
	100	61.00±3.92	54.80±2.71*	59.40±9.86	60.60 ± 3.12
	PC	54.60±5.06	53.60±2.32	54.60±11.30	53.60 ± 2.32
HETEROP HILS (%)	NC	41.40±7.09	46.40±0.93	41.40±7.09	46.40 ± 0.93*
	6.25	44.33±3.06	39.00±2.39	33.80± 3.70	30.20 ±3.81
	12.5	35.00±7.31	36.60±3.33*	37.80± 6.61	29.60 ± 1.63
	25	46.00±6.44	38.00±2.41	28.00±4.90*	23.40 ± 2.16*
	50	45.60±6.19	35.80±2.60*	46.00±6.24	27.00 ± 3.08*
	100	33.25±7.80	38.00±2.41	34.60±9.07	31.80 ± 3.95
	PC	37.80±5.00	39.60±2.71	37.80±11.19	39.60 ± 2.71
MONOCY TES (%)	NC	2.20±1.30	3.40±0.68	2.20±1.30	3.40 ± 0.68
	6.25	3.33±2.08	4.80±0.20	3.20±1.10	4.60 ± 0.24
	12.5	2.40±0.89	3.80±0.37*	4.40±1.14	3.00 ± 0.71
	25	5.20±0.84*	3.20±0.37	4.20±0.84	3.20 ± 0.37
	50	4.00±1.00	3.20±0.80	4.33±0.58	2.80 ± 0.73
	100	2.00±0.82	3.20±0.37	3.80±1.10	4.00 ± 0.45*
	PC	3.80±1.92	2.60±0.51	3.80±1.92	2.60 ± 0.50
EOSINOP HILS (%)	NC	4.40±0.55	5.20±0.58	4.40±0.55	5.20 ± 0.58
	6.25	3.00±1.00	4.20±0.37	2.60±1.34	2.40 ± 0.93
	12.5	3.40±2.07	2.60±0.51	3.00±1.58	3.20 ± 0.80
	25	2.60±1.34	3.80±0.37	3.20±0.45	3.80 ± 0.49
	50	3.00±0.71	3.60±0.75	5.00±1.00	3.60 ± 1.17
	100	3.25±2.06	3.80±0.37	1.80±1.30*	3.40 ± 0.81

PC 3.20±1.30 3.80±0.73 3.20±1.30 3.80 ± 0.73

Table 4.13. Red blood cell (RBC) indices and Red cell indices of *Clarias gariepinus* exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days

Parameters	Concentrations	Exposure periods		Table 4.14.
		7 days	28 days	
PCV	NC	34.00 ± 2.88	35.80± 2.08	White blood cells count and differential of <i>Clarias gariepinus</i> exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days
	6.25	27.40 ± 2.46	23.80± 1.85	
	12.5	20.40 ± 1.72*	20.80± 1.16	
	25	23.00 ± 1.41*	26.80± 1.07	
	50	28.80 ± 2.56	24.00± 1.05*	
	100	24.20 ± 1.36*	21.50± 1.44	
	PC	24.60 ± 3.61	31.20± 3.61	
Hb(g/dl)	NC	11.32 ±0.98	12.32±0.57	White blood cells count and differential of <i>Clarias gariepinus</i> exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days
	6.25	9.24 ±.84	8.08± 0.69	
	12.5	6.76 ±.64*	6.80± 0.29	
	25	7.62 ±.39*	8.84± 0.25	
	50	9.14 ±.87	7.98± 0.44*	
	100	7.76 ±.39*	7.25± 0.43	
	PC	7.98 ±1.62	10.34± 1.34	
RBC (x 10 ⁶)	NC	3.55 ± 0.21	3.77± 0.10	White blood cells count and differential of <i>Clarias gariepinus</i> exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days
	6.25	3.29 ± 0.37	2.24± 0.33	
	12.5	1.98 ± 0.44*	1.63± 0.05	
	25	2.00 ± 0.36*	3.34± 0.25*	
	50	3.01 ± 0.37	2.25± 0.43*	
	100	2.29 ± 0.37	1.94± 0.27	
	PC	2.43 ± 0.35	3.43± 0.35	
MCHC (%)	NC	33.27 ± 0.244	34.62± 1.51	White blood cells count and differential of <i>Clarias gariepinus</i> exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days
	6.25	33.70 ± 0.14	33.89± 0.53	
	12.5	33.08 ± 0.78	32.80± 0.57	
	25	33.24± 0.76	33.08± 0.78	
	50	31.74 ± 0.68	33.20± 0.49*	
	100	32.12 ± 0.43	33.78± 0.34	
	PC	32.54 ± 1.30	32.99± 0.72	
MCH(pg)	NC	31.84 ± 1.51	32.67± 0.91	White blood cells count and differential of <i>Clarias gariepinus</i> exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days
	6.25	28.58 ±1.34	37.51± 2.72	
	12.5	36.86 ±3.33	42.09± 2.84	
	25	40.90 ±4.62	27.02± 1.79*	
	50	31.71 ±3.25	38.83± 4.33	
	100	36.54 ±4.16	38.44± 2.94	
	PC	35.34 ±7.69	30.26± 2.22	
MCV(fl)	NC	95.66 ± 4.31	94.78± 3.28	White blood cells count and differential of <i>Clarias gariepinus</i> exposed to 1:1 mixture of CuO NPs and Ag NPs for 7 and 28 days
	6.25	84.88 ± 4.32	110.66± 7.65	
	12.5	112.05 ± 11.15	129.01± 10.71*	
	25	122.23 ± 11.86	81.38± 3.65	
	PC	99.37 ± 8.43	117.61± 14.08	

NC- Negative control (Tap water) PC Positive control (0.08 ml/l Benzene) *Values significant at p < 0.05

WBC (x10 ³)	NC	32.27 ± 3.61	24.190± 2.97
	6.25	23.80 ±3.29	16.430± 2.02
	12.5	25.29±2.39	18.900± 1.78
	25	17.13±1.81*	16.540± 2.48*
	50	15.09±2.24*	17.160± 1.20*
	100	15.36±1.76*	14.963± 1.74*
	PC	19.42 ±8.18	24.550± 1.11
	PLATELETS (x 10 ⁶)	NC	0.28 ±0.05
6.25		0.28 ±0.04	20.88± 0.03
12.5		0.32±0.02	21.30± 0.19
25		0.18±0.02	23.58± 0.03*
50		0.15±0.02*	28.78± 0.02*
100		0.14±0.08*	24.80± 0.05*
PC		0.27±0.10	25.92± 0.03
LYMPHOCYTS (%)		NC	51.80 ± 2.96
	6.25	62.40 ±1.66*	56.80± 3.98*
	12.5	55.20 ±1.59	49.80± 1.88*
	25	59.40 ±2.38	53.00± 2.07*
	50	61.80 ±2.65*	52.60± 1.91*
	100	59.60 ±2.69	58.75 ± 2.02*
	PC	54.60 ± 5.60	53.60± 2.32
	HETEROPHILS (NEUTROPHILS) (%)	NC	41.40 ± 3.17
6.25		31.00 ±1.87*	36.20± 3.87
12.5		38.60 ±1.50	42.40± 1.78
25		30.80 ±2.00*	45.40± 4.50
50		31.00 ±3.21*	47.40± 5.11
100		32.40 ±2.58	32.50± 1.44
PC		37.80 ± 5.00	39.60± 2.71
MONOCYTES (%)		NC	2.20 ± 0.58
	6.25	3.00 ± 0.71	3.20± 0.58
	12.5	2.20 ± 0.73	3.40± 0.81
	25	3.80 ± 0.37	3.20± 0.66
	50	2.80 ± 0.66	3.80± 0.97
	100	4.60 ± 0.51*	4.50± 0.29
	PC	3.80 ± 1.92	2.60± 0.51
	EOSINOPHILS (%)	NC	4.40 ± 0.24
6.25		3.60 ± 0.75	3.60± 1.03
12.5		3.80 ± 0.86	4.00± 0.89
25		3.40 ± 0.40	4.00± 0.89
50		3.20 ± 0.86	4.00± 0.63
100		3.00 ± 0.71	4.00± 0.41
PC		3.20 ± 1.30	3.80± 0.73

NC- Negative control (Tap water) PC: Positive control 0.005% Benzene *Statistically significant (p< 0.05)

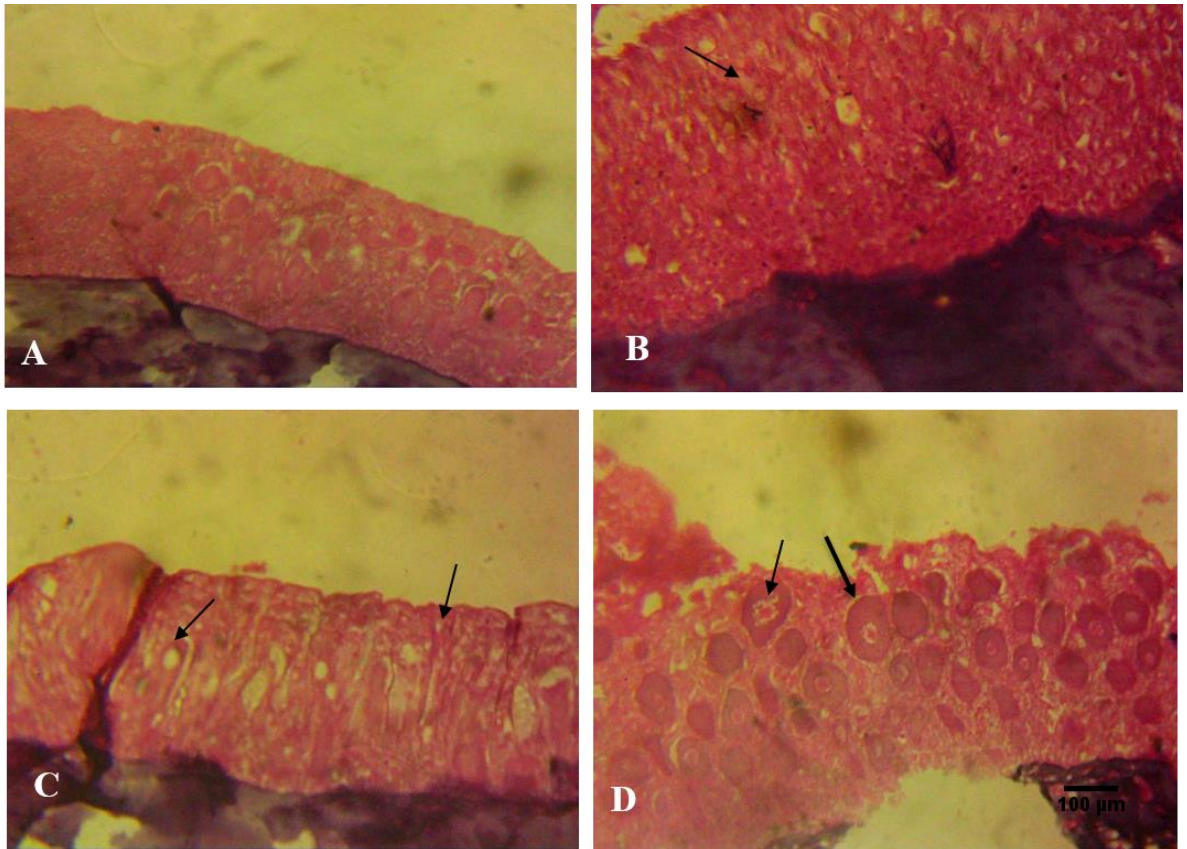


Figure 4.22. Histological changes in epidermal skin layer in juvenile *C. gariepinus* exposed for 28 days to AgNPs, CuONPs, 1:1 mixture (H and E; x 400)

(A) Negative control showing no visible lesion

(B) moderate hyperplasia of keratinocytes (arrow) at 6.25 mg/L AgNPs

(C) moderate hyperplasia of keratinocytes and goblet cells (arrows) at 100 mg/L CuONPs

(D) marked hyperplasia of alarm cells (arrow) at 100 mg/L 1:1 mixture of NPs, x 400

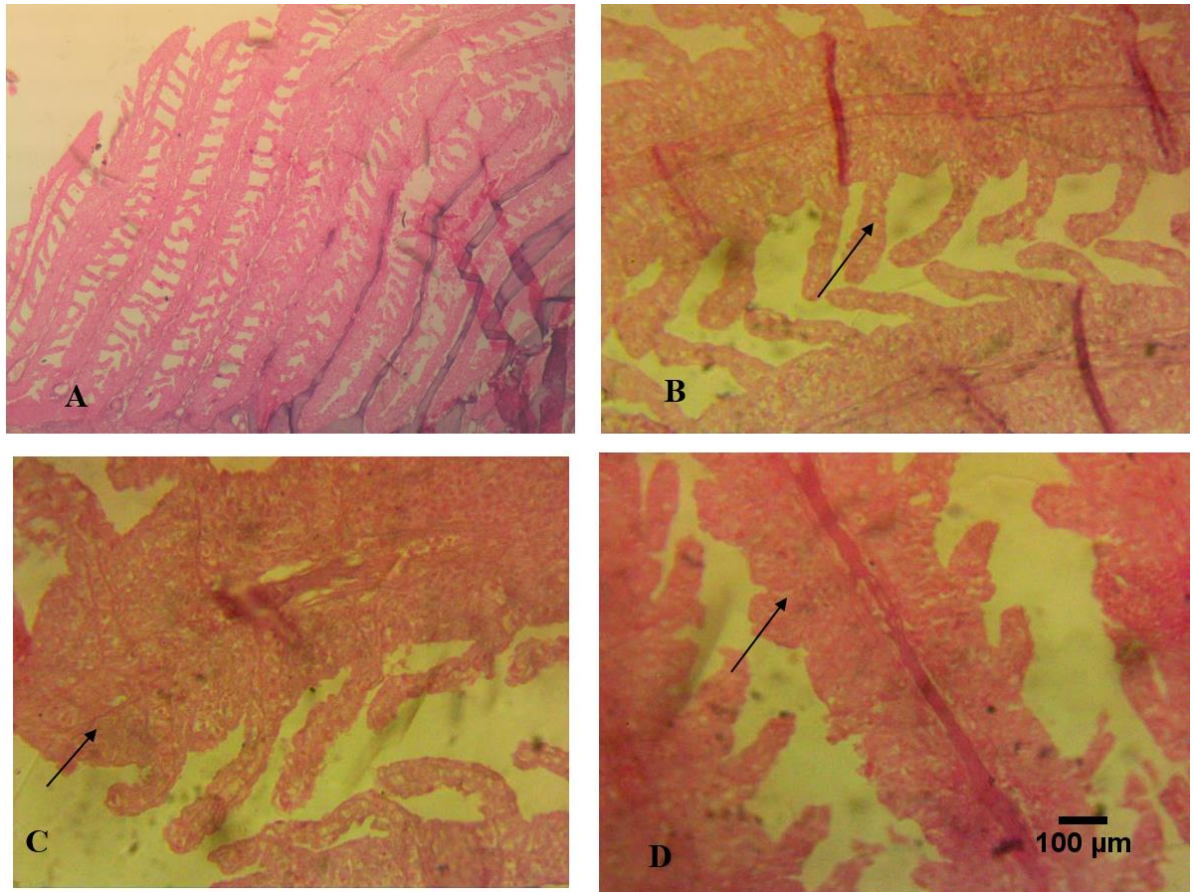


Figure 4.23. Histological changes in the gills of juvenile *C. gariepinus* exposed to AgNPs, CuONPs and 1:1 mixture. (H and E; x 400)

Gills with:

(A) numerous and tall filaments, the primary and secondary gill lamella appear normal and distinct in Negative control group.

(B) moderate lamella hyperplasia (arrow) at 100 mg/L AgNPs

(C) moderate lamella hyperplasia (arrow) at 25 mg/L CuONPs

(D) moderate lamella hyperplasia (arrow) and marked clumping and sloughing off of the secondary gill lamella at 50 mg/L 1:1 mixture.

all at X 400 except (A)

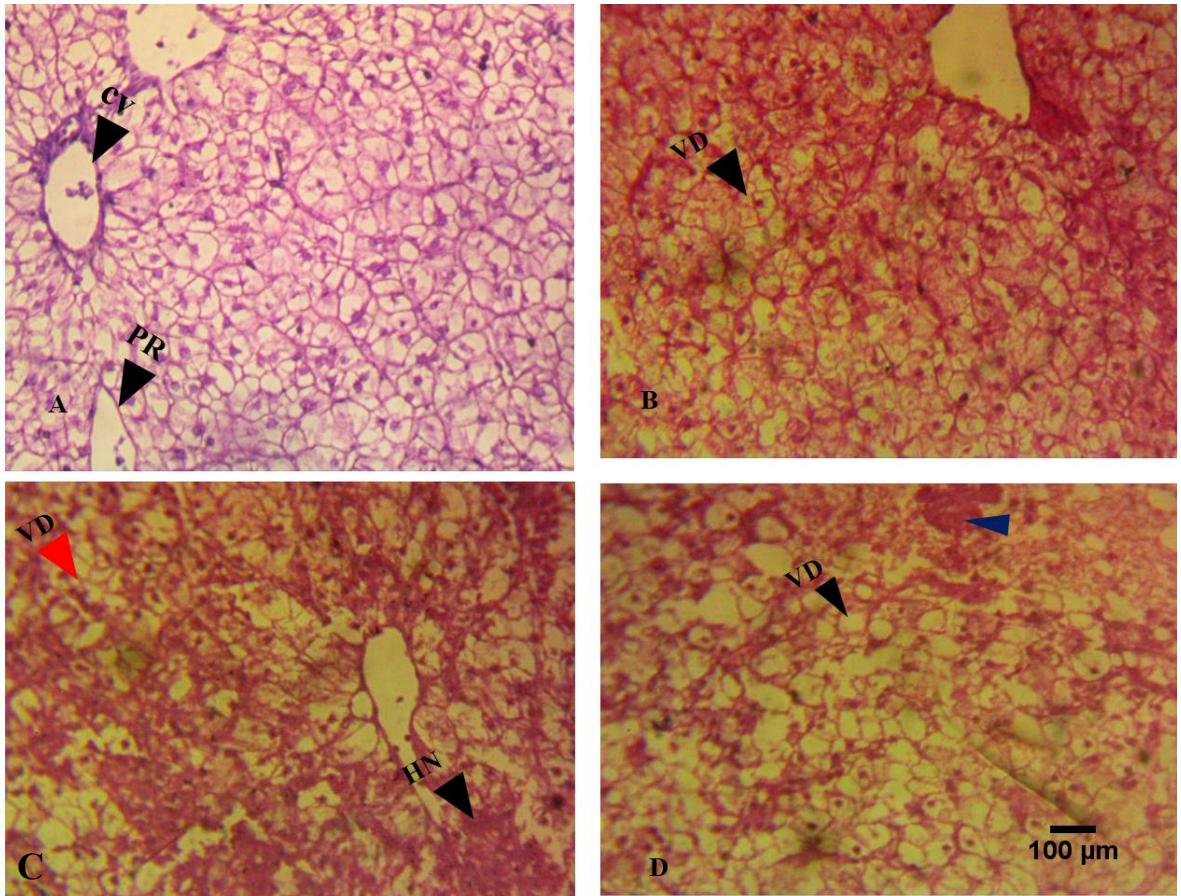


Figure 4.24. Histological alterations in the liver of juvenile *C. gariepinus* exposed to AgNPs, CuONPs, 1:1 mixture for 28 days (H and E; x 400)

- (A) Negative control: normal hepatocytes having large clear cytoplasmic appearance with the nucleus centrally placed; CV: Central Vein, PV: Periportal region
- (B) Diffuse vacuolar degeneration (VD) of hepatocytes (arrows head) at 6.25 mg/L AgNPs
- (C) Diffuse vacuolar degeneration (VD) (red arrow head) of hepatocytes and foci of hepatocellular necrosis (HN) (black arrow head) at 50 mg/L CuONPs.
- (D) Vacuolar degeneration (black arrow) of hepatocytes at the centrilobular region and vacuolar degeneration (blue arrow head) at 25 mg/L 1:1 mixtures

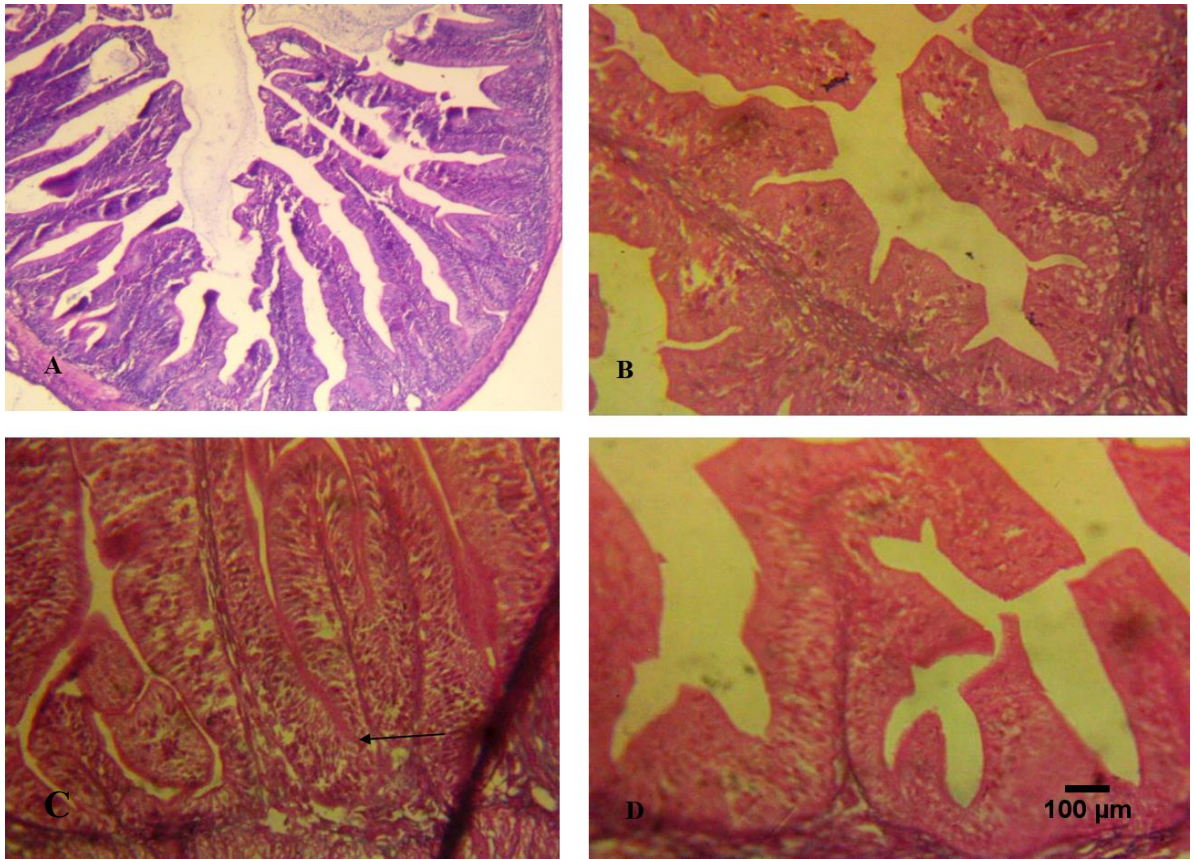


Figure 4. 25. Histology of the intestine of juvenile *C. gariepinus* exposed to AgNPs, CuONPs and 1:1 mixture for 28 days (H and E; x 400)

- (A) Negative control group
- (B) No observable lesion in the AgNPs groups
- (C) Moderate cryptal hyperplasia (arrow) at 25 mg/L CuONPs
- (D) No observable lesion in the 1:1 mixture group

Table 4.15. Effect of AgNPs, CuONPs and 1:1 mixture on the levels of oxidative stress biomarkers in *C. gariepinus* following 28 days exposure

Concentrations (mg/L)		MDA (unit/mg protein)	GSH (unit/mg protein)	SOD(unit/mg protein)	CAT(μ mol H ₂ O ₂ consumed/min/mg protein)
Ag NPs	NC	15.91 \pm 2.50	774.3 \pm 150.4	0.1658 \pm 0.01	120.1 \pm 43.74
	6.25	7.276 \pm 2.39	240.7 \pm 26.05*	0.0906 \pm 0.01	39.67 \pm 10.93*
	12.5	28.35 \pm 17.1	481.2 \pm 144.6	0.07609 \pm 0.03	119.2 \pm 4.10
	25	18.22 \pm 1.97	912 \pm 224.4	0.3689 \pm 0.08***	141 \pm 29.4
	50	24.26 \pm 2.37	1127 \pm 166.7	0.3014 \pm 0.04**	80.28 \pm 0.60
	100	15.65 \pm 1.79	778.3 \pm 141.4	0.1526 \pm 0.03	87.06 \pm 18.09
CuO NPs	NC	15.91 \pm 2.50	774.3 \pm 150.4	0.1658 \pm 0.01	120.1 \pm 43.74
	6.25	20.12 \pm 2.7	1257 \pm 136.3**	0.3493 \pm 0.03***	41.11 \pm 4.84**
	12.5	20.42 \pm 3.18	1143 \pm 130.4*	0.1922 \pm 0.04	12.06 \pm 2.21***
	25	37.86 \pm 2.5***	845.6 \pm 174.8	0.1789 \pm 0.02	17.25 \pm 0.00***
	50	16.23 \pm 2.2	840 \pm 224.3	0.2747 \pm 0.02**	3.908 \pm 2.22***
	100	29.06 \pm 1.25***	1244 \pm 63.1**	0.2523 \pm 0.05*	3.137 \pm 2.56***
Mixture	NC	15.91 \pm 2.50	774.3 \pm 150.4	0.1658 \pm 0.01	120.1 \pm 43.74
	6.25	13.39 \pm 2.9	629.5 \pm 77.83	0.1052 \pm 0.07	88 \pm 8.93
	12.5	13.83 \pm 3.2	784.3 \pm 178.3	0.08154 \pm 0.02*	80.42 \pm 11.39
	25	5.682 \pm 0.18*	285.6 \pm 79.84*	0.07482 \pm 0.03**	53.13 \pm 0.00
	50	14.85 \pm 3.97	622.6 \pm 162.4	0.1349 \pm 0.04	50.77 \pm 6.42*
	100	7.326 \pm 1.65*	696.9 \pm 240.7	0.08106 \pm 0.02*	85.01 \pm 20.95

NC = Negative control (Dechlorinated Tap water) *Significant at $p < 0.05$ Values are expressed as mean \pm SE

Table 4. 16. Interaction Factor of AgNPs and CuONPs mixture (1:1) hepatic oxidative stress biomarkers in juvenile *Clarias gariepinus* following 28 days exposure

Conc. (mg/L)	†Interaction factor (IF± SE _{IF})			
	MDA	GSH	SOD	CAT
NC	-	-	-	-
6.25	1.904± 2.84	-93.9 ± 88.9	-0.17 ± 0.05	128.22 ± 9.35
12.50	-19.03± 12.37	-65.6 ±152.4	-0.02 ± 0.03	70.16 ± 7.29
25.00	-34.49±1.84	-697.7 ±149.52	-0.31 ±0.05	15.88± 20.79
50.00	-9.73± 2.95	-570.1 ± 186.63	-0.28 ±0.03	87.58 ± 4.73
100.00	-21.47± 1.64	-551.1± 186.99	-0.16 ±0.03	115.81 ±19.62

NC = Negative control (Dechlorinated Tap water) PC = positive control (0.05 mL/L Benzene)

† NB: Negative IF value = antagonistic interaction; positive IF = synergistic interaction

Analysis of interaction factor (Table 4.16) of the NP 1:1 mixture on oxidative stress parameters reflected an antagonistic effect on MDA, GSH, and SOD and a synergistic effect on CAT activity.

4.4 Cytogenotoxic and systemic toxicity induced by silver, copper(II) oxide nanoparticles and their 1:1 mixture in mice

4.4.1 Acute toxicity of Silver and Copper(II) oxide nanoparticles in mice

No mortality was observed at the tested concentrations (600 mg/kg) of both AgNPs and CuONPs following the administration of a single oral dose. There was no observable behavioral within the 14-day period except decrease in body weight in the AgNPs group. Therefore, the LD₅₀ of both NPs were concluded to be higher than 600 mg/kg and a concentration of 300 mg/kg and below was selected for the sub lethal toxicity

4.4.2 Micronuclei induced by AgNPs, CuONPs and their 1:1 mixtures in mice

Figures 4.26 – 4.28 shows frequencies of micronucleated polychromatic erythrocytes (MNPCE) in the bone marrow of mice orally administered AgNPs, CuONPs and their 1:1 mixture for 14 days and 28 days. Following a one – way ANOVA analysis, AgNPs significantly ($p < 0.05$) induced MNPCE at 150 mg/kg and 300 mg/kg compared with the negative control (Figure 4.26). There was no significant difference ($p > 0.05$) in the frequency of MNPCE induced at both 14 and 28 - day exposure periods.

On the other hand, CuONPs induced MNPCE at 150 mg/kg and 300 mg/kg at 14 days but the frequency of MNPCEs decreased at 28 – day exposure. Two - way ANOVA showed significant ($p < 0.05$) reduction in frequencies of MNPCE induced at the two highest concentrations at 28th day (4.27).

The 1:1 mixture significantly induced the formation of MNPCEs at 18.75, 37.5 and 300 mg/kg bw compared to the control group at 14 – day exposure period but at 28 - day exposure period, a significant increase in MNPCEs was recorded at all concentrations (Figure 4.28). Two – way ANOVA showed that the observed MNPCE frequencies at 18.75 – 150 mg/kg bw for both exposure periods were significantly

different ($p < 0.05$). Figures 4.32 shows the normal PCE, NCE and MNPCEs observed in the bone marrow of mice exposed to NPs.

The cytotoxicity indices of AgNPs, CuONPs and their 1:1 mixture on the bone marrow erythrocytes are presented in Figures 4.29 – 4.31. Cytotoxic effect determined by PCE to NCE ratio showed that AgNPs stimulated the production of higher %PCE (Figure 4.29) at the 3 lowest concentrations (18.75, 37.5 and 75 mg/kg) at 14 – day experiment but not at 28 – exposure period. The frequency of % PCE significantly decreased between 14 and 28 – day exposure period.

There was no significant difference in frequencies of % PCE in the bone marrow of CuONPs exposed mice at 14 but cytotoxicity was observed at 28 – day exposure period; the frequency of %PCE decreased significantly ($p < 0.05$) at all concentrations except at 75 mg/kg bw showing that frequency % NCE increased (Figure 4.30).

The 1:1 mixture also induced an increase in % PCE at 18.75 and 37.5 mg/kg following 14- day treatment. However, the % frequency of PCEs in the bone marrow decreased significantly ($p < 0.05$) at the 28 day – exposure period (Figure 4.31).

Interactive effect of the 1:1 mixture on MNPCEs and cytotoxicity (PCE to NCE ratio) is presented in Table 4.17. Interaction factor analysis showed that AgNPs and CuONPs had antagonistic effect indicated by the negative IF value at the three higher concentrations at both 14 and 28 – day exposure periods. Similarly, at 14 days exposure, the 1:1 mixture of the NPs had an antagonistic effect on PCE/NCE ratio at the three higher concentrations and a synergistic effect. at the 28 days exposure period except at 150 mg/kg.

4.4.3 Body and relative organ weight change

There were considerable changes in body and relative organ weight of mice orally administered AgNPs, CuONPs and their 1:1 mixture are shown in Tables 4.18 – 4.20. There were increases in body weight of AgNPs - exposed mice at 14 and 28 - exposure periods except mice administered the highest dose of 300 mg/kg AgNPs at the 14 days exposure period (Table 4.18). The relative liver, kidney and spleen weight of exposed mice increased at 14th day but the relative testis weight decreased. There was a significant four - fold increase in spleen weight at 150 mg/kg. However, at the 28th

day there were increases in all the relative organ weights (liver, kidney, spleen and testis) which was only significant at 300 mg/kg compared to the negative control.

The body weight of the CuONPs exposed mice increased at both 14 and 28 – day exposure periods compared to the negative control (Table 4.19). At 14 days there was a hypertrophy (increase) in relative liver and spleen weight compared to the control. Similarly, relative kidney weight increased at 18.75, 75 and 300 mg/kg but atrophy (decrease) was observed at 37.5 mg/kg and 150 mg/kg bw. However, relative testis weight decreased across the concentrations. At the 28 – day exposure period, the organ weight of the CuONPs mice increased with significant renal and hepatosplenomegaly at 37.5 mg.kg compared to the control.

In mice exposed to the 1:1 mixture, there were increases in body weight at both 14 and 28 – day exposure periods except at 75 mg/kg bw compared to the controls. At the 14 – day exposure period, the 1:1 mixture induced a significant increase in the relative liver weight compared to the control; the mixture (1:1) also induced increase in relative kidney weight at 37.5, 75.00 and 300 mg/kg and a decrease 18.75 and 150 mg.kg compared to the control but this was not significant ($p > 0.05$). Also, non-significant decrease (at 18.75, 75.0 and 300 mg/kg) and increase (at 37.5 and 150 mg/kg) in spleen weight were observed were compared with the control. The 1:1 mixture also induced atrophy of testis compared to the negative control group at all concentration except at 75 mgk.kg⁻¹.

4.4.4 Haematological changes in mice

Variations in the RBC and its indices in mice treated with the nanoparticles and their 1:1 mixture are shown in Tables 4.21 to 4.23. At the 14 – day exposure period, mice exposed to AgNPs exhibited decreases in PCV, Hb, RBC, MCH, MCHC, and a concomitant increase in MCV compared to the control. At the 28th day, AgNPs induced a significant ($p < 0.05$) decrease in PCV at 18.75 and 75 mg/kg bw; and a concomitant increase in Hb and RBC compared to the negative control (Table 4.21).

For the 14 – day exposure period, CuONPs – exposed mice showed a non-significant ($p < 0.05$) decrease in PCV, Hb, RBC, MCHC and MCV compared to the negative control. Following 28 - day exposure, CuONPs induced an increase in PCV, MCHC,

MCH and a significant ($p < 0.05$) increase in Hb and RBC at 75.0 mg/kg and a concomitant decrease in MCV (Table 4.21).

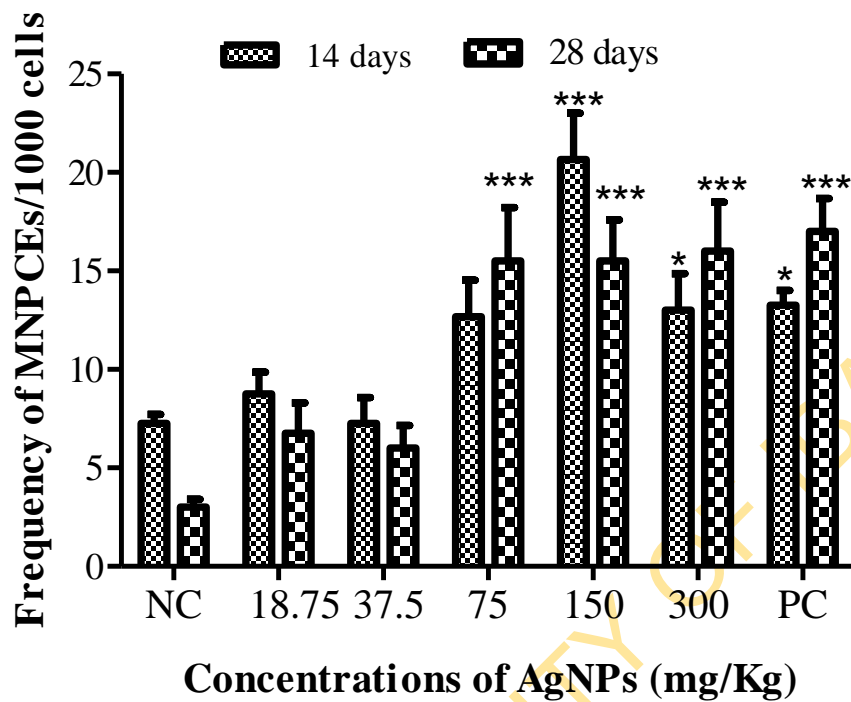


Figure 4.26. Frequencies of MNPCE induced in mice orally administered AgNPs for 14 and 28- day exposure periods

NC- Negative control (dechlorinated tap water); PC – Positive control (Cyclophosphamide - 20 mg/kg body weight)

Using one way and two-way ANOVA * $p < 0.05$, *** $p < 0.001$.

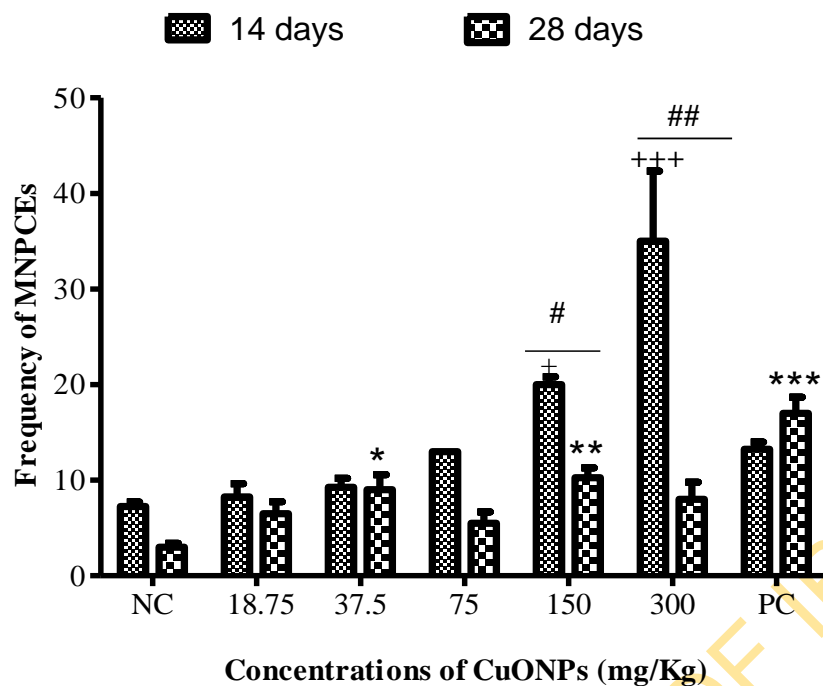


Figure 4. 27. Frequencies of MNPCE induced in mice orally administered CuONPs for 14 and 28-day exposure periods

NC- Negative control (dechlorinated tap water); PC – Positive control (Cyclophosphamide - 20 mg/kg body weight)

Following a one-way analysis of variance (ANOVA), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ at 28 days compared with corresponding control. Using two-way ANOVA # $p < 0.05$, ## $p < 0.01$ between 14 and 28 days exposure periods.

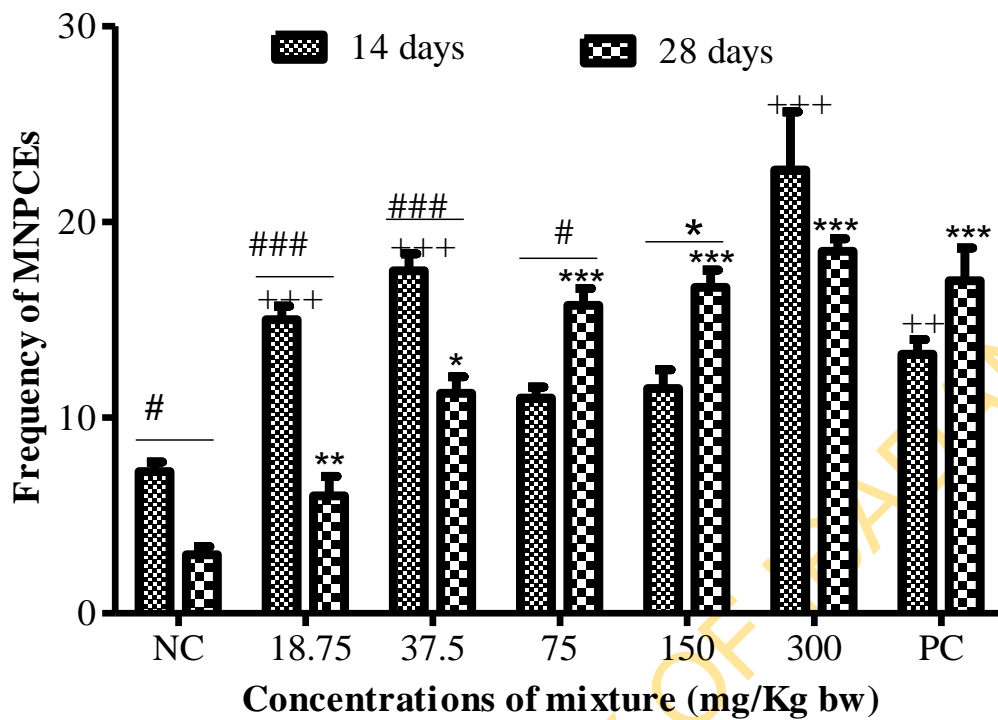


Figure 4.28. Frequencies of MNPCE induced in mice orally administered 1:1 mixture of AgNPs and CuONPs for 14 and 28- day exposure periods

NC- Negative control (dechlorinated tap water); PC – Positive control (Cyclophosphamide - 20 mg/kg body weight)

Following a one-way analysis of variance (ANOVA), ++ $p < 0.01$, +++ $p < 0.001$ at 14 days and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ at 28 days compared with corresponding control. Using two-way ANOVA, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ between 14 and 28 days exposure periods.

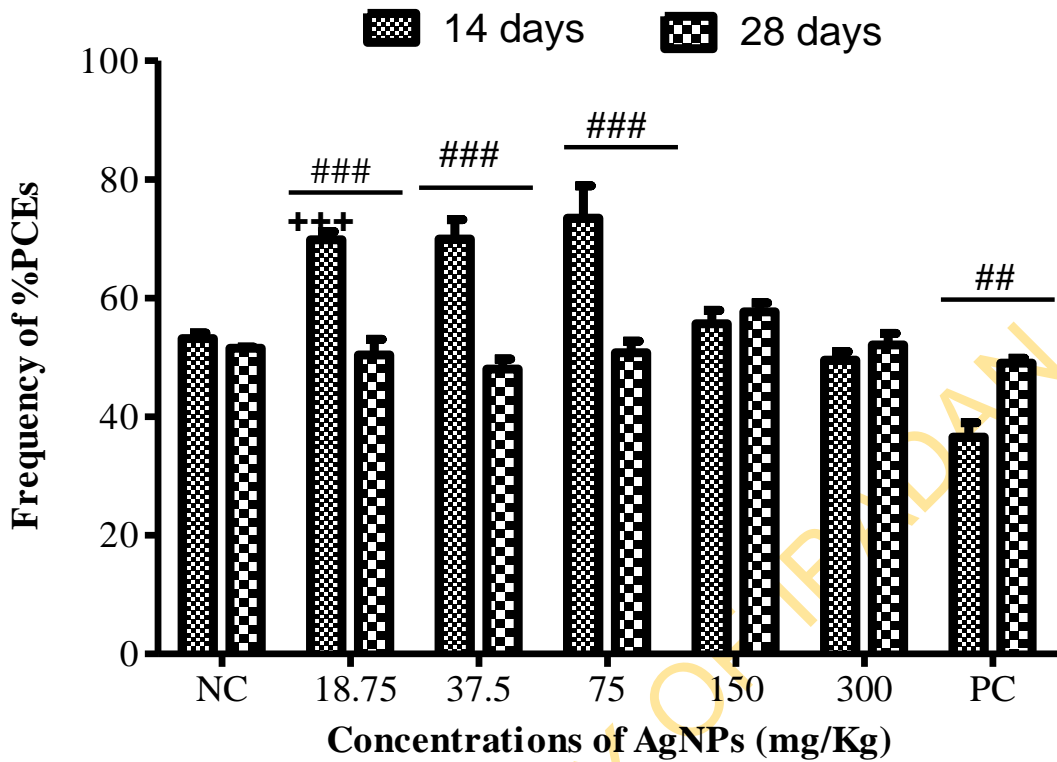


Figure 4.29. Frequencies of %PCE induced in mice orally administered AgNPs for 14 and 28 -day exposure periods

NC- Negative control (dechlorinated tap water); PC - Cyclophosphamide (20 mg/kg body weight)

Following a one-way analysis of variance (ANOVA), ^a p< 0.05, ^b p< 0.01, ^c p< 0.001 at 14 days and ⁺ p< 0.05, ⁺⁺ p< 0.01, ⁺⁺⁺ p< 0.001 at 28 days compared with corresponding control. Using two-way ANOVA ^{*} p< 0.05, ^{**} p< 0.01, ^{***} p< 0.001 between 14 and 28 days exposure periods.

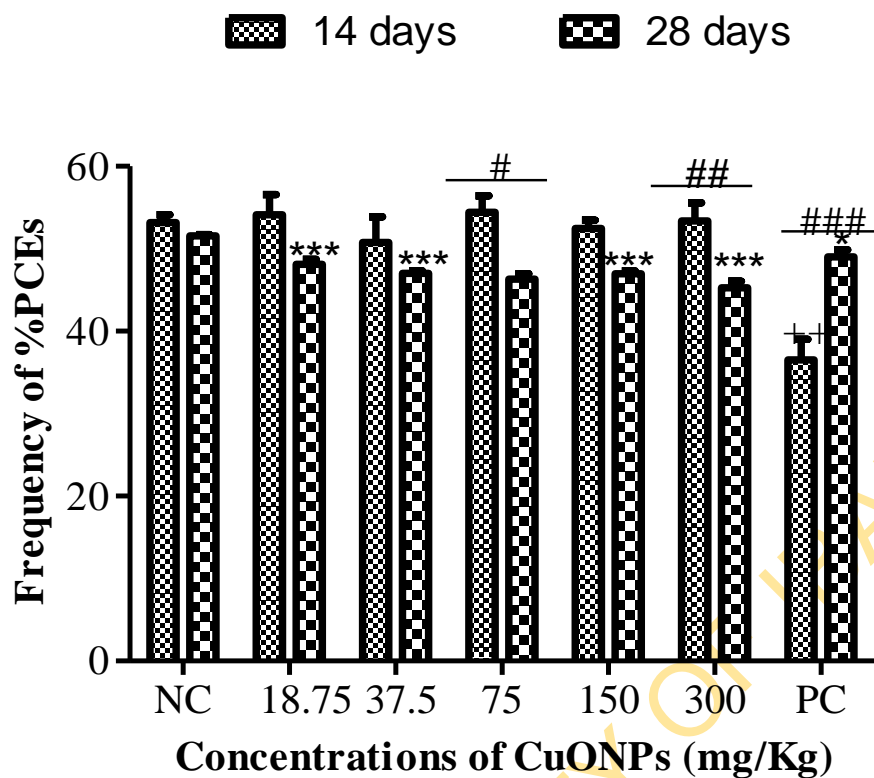


Figure 4.30. Frequencies of %PCE induced in mice orally administered CuONPs for 14 and 28 -day exposure periods.

NC- Negative control (dechlorinated tap water); PC - Cyclophosphamide (20 mg/kg body weight)

Following a one-way analysis of variance, ⁺⁺ $p < 0.01$ at 14 days and ^{***} $p < 0.001$ at 28 days compared with corresponding control. Using two-way ANOVA [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ between 14 and 28 days exposure periods.

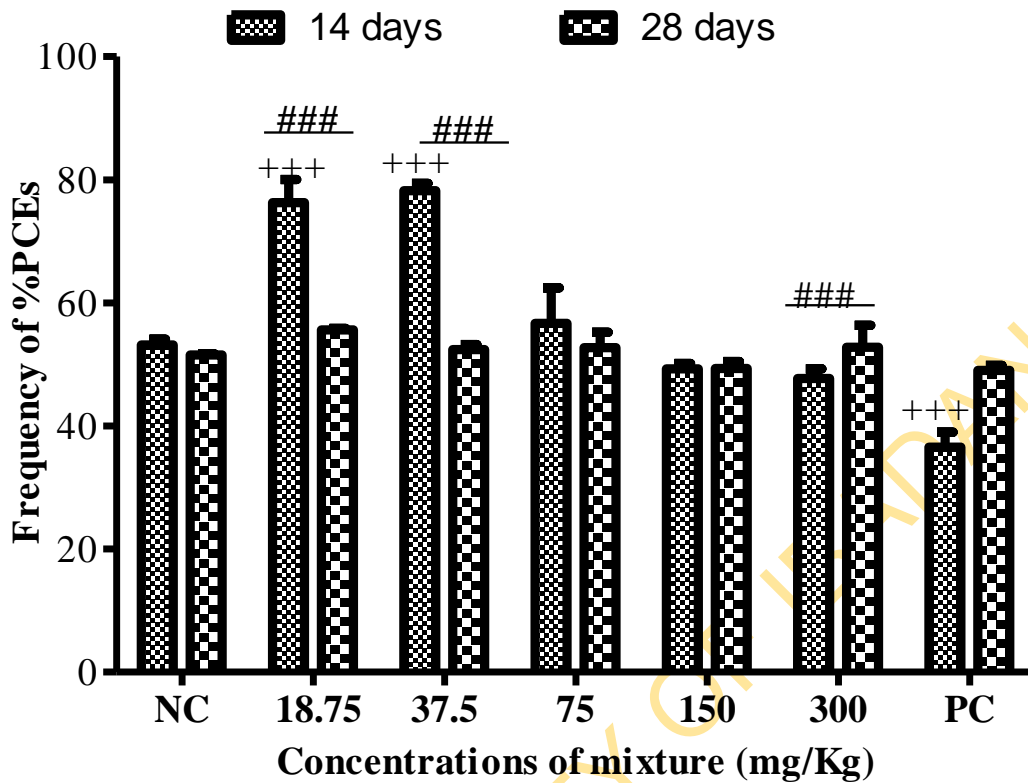


Figure 4.31. Frequencies of %PCE induced in mice orally administered 1:1 mixture of AgNPs and CuONPs for 14 and 28- day exposure periods

NC- Negative control (dechlorinated tap water); PC - Cyclophosphamide (20 mg/kg body weight)

Following a one-way analysis of variance, $+++)$ $p < 0.001$ at 14 days compared with corresponding control. Using two-way ANOVA $###$ $p < 0.001$ between 14 and 28 days exposure periods.

Table 4.17. Interactive effects of 1:1 mixture of AgNPs, CuONPs on frequency of MNPCEs and cytotoxicity index (%PCE to NCE) in bone marrow of mice after 14- and 28-days exposure periods

Concentrations (mg/Kg bw)	Interaction factor (IF± SE _{IF})	
	MNPCEs	Cytotoxicity Index
14 days exposure		
NC	7.25 ± 0.48	53.19 ± 0.93
18.75	5.25 ± 1.90	5.48 ± 4.65
37.5	8.25 ± 1.83	10.71 ± 4.67
75.00	-7.45 ± 1.95	-18.07 ± 8.17
150.00	-21.95 ± 2.65	-5.68 ± 2.60
300.00	-18.08 ± 8.14	-1.93 ± 3.03
28 days exposure		
NC	3.00 ± 0.41	51.56 ± 0.15
18.75	2.89 ± 3.26	8.61 ± 2.70
37.5	-0.75 ± 2.14	8.85 ± 1.87
75.00	-2.25 ± 3.09	7.13 ± 3.25
150.00	-6.08 ± 2.50	-3.73 ± 1.91
300.00	-2.5 ± 3.12	6.97 ± 4.14

NB: IF = (AgNP+CuONPs - Control) - [(AgNPs - Control) + (CuONPs - Control)] = (AgNP+CuONPs - AgNPs - CuONPs + Control)

Table 4.18. Body weight changes and relative organ weight of mice orally administered AgNPs following 14 and 28 -day exposure periods

	Conc (mg/kg)	Initial body weight (g)	Final body weight (g)	% change in body weight	Relative organ weight (%)			
					Liver	Kidney	Spleen	Testis
14 days	NC	25.4 ± 3.98	26.10 ± 2.10	2.76	4.60 ± 0.20	1.51 ± 0.10	0.53 ± 0.07	0.76 ± 0.06
	18.75	23.48 ± 2.53	26.35 ± 2.29	12.24	5.57 ± 0.13	1.521 ± 0.05	0.70 ± 0.03	0.61 ± 0.02
	37.5	24.65 ± 0.97	25.25 ± 2.04	2.43	5.93 ± 0.46	1.534 ± 0.12	0.73 ± 0.13	0.74 ± 0.06
	75	23.67 ± 1.61	24.00 ± 1.30	1.41	4.78 ± 0.33	1.60 ± 0.08	0.45 ± 0.02	0.65 ± 0.04
	150	21.28 ± 1.10	25.30 ± 0.98	18.92	4.86 ± 0.33	1.60 ± 0.06	2.35 ± 1.01*	0.73 ± 0.04
	300	27.48 ± 2.10	22.88 ± 2.35	-16.74	5.51 ± 0.26	1.38 ± 0.03	0.89 ± 0.11	0.50 ± 0.27
	PC	23.68 ± 0.73	23.05 ± 0.82	-2.64	4.92 ± 0.59	1.69 ± 0.08	0.81 ± 0.10	0.82 ± 0.10
28 days	NC	25.15 ± 0.99	28.08 ± 3.23	1.42	4.72 ± 0.44	1.46 ± 0.12	0.43 ± 0.04	0.66 ± 0.05
	18.75	22.58 ± 1.72	28.55 ± 2.22	26.47	5.51 ± 0.18	1.54 ± 0.12	0.84 ± 0.08	0.85 ± 0.02
	37.5	24.15 ± 1.54	27.35 ± 1.03	13.25	5.28 ± 0.26	1.73 ± 0.07	0.56 ± 0.06	0.72 ± 0.11
	75	20.65 ± 1.05	25.65 ± 2.20	20.85	5.53 ± 0.49	1.81 ± 0.18	0.86 ± 0.11	0.81 ± 0.08
	150	21.05 ± 1.73	26.43 ± 0.99	23.28	5.74 ± 0.42	1.59 ± 0.05	0.96 ± 0.27	0.76 ± 0.03
	300	21.60 ± 2.36	25.30 ± 3.07	17.12	6.18 ± 0.18	2.13 ± 0.43	0.90 ± 0.28	0.71 ± 0.03
	PC	25.43 ± 1.64	25.25 ± 2.43	-0.82	5.19 ± 0.21	1.49 ± 0.06	0.68 ± 0.14	0.82 ± 0.05

NC = Negative control (distilled water) *Significant at $p < 0.05$

Table 4.19. Body weight changes and relative organ weight of mice orally administered CuONPs following 14 and 28- day exposure periods

	Conc (mg/kg)	Initial body weight (g)	Final body weight (g)	% change in body weight	Relative organ weight (%)			
					Liver	Kidney	Spleen	Testis
14 days	NC	25.4 ± 3.98	26.10 ± 2.10	2.76	4.60 ± 0.20	1.51 ± 0.10	0.53 ± 0.07	0.76 ± 0.06
	18.75	20.65 ± 0.65	22.13 ± 1.33	7.14	4.65 ± 0.05	1.59 ± 0.12	0.53 ± 0.081	0.76 ± 0.05
	37.5	23.13 ± 1.33	24.28 ± 3.87	4.97	5.91 ± 0.09	1.42 ± 0.02	0.61 ± 0.49	0.72 ± 0.69
	75	23.45 ± 1.97	25.50 ± 2.72	8.74	5.13 ± 0.60	1.53 ± 0.10	0.72 ± 0.10	0.69 ± 0.04
	150	23.22 ± 3.68	23.63 ± 2.00	1.72	5.32 ± 0.33	1.40 ± 0.19	0.69 ± 0.23	0.70 ± 0.05
	300	22.33 ± 1.98	24.10 ± 2.34	7.95	5.51 ± 0.26	1.60 ± 0.02	0.65 ± 0.18	0.75 ± 0.06
	PC	23.68 ± 0.73	23.05 ± 0.82	-2.64	4.92 ± 0.59	1.69 ± 0.08	0.81 ± 0.10	0.82 ± 0.10
28 days	NC	25.15 ± 0.99	28.08 ± 3.23	1.42	4.72 ± 0.44	1.46 ± 0.12	0.43 ± 0.04	0.66 ± 0.05
	18.75	21.95 ± 1.86	26.95 ± 3.37	28.32	5.25 ± 0.14	1.51 ± 0.07	0.67 ± 0.14	0.70 ± 0.04
	37.5	23.03 ± 1.18	26.05 ± 1.65	13.09	6.547 ± 0.21**	1.878 ± 0.06**	1.433 ± 0.14**	0.74 ± 0.04
	75	23.45 ± 1.97	25.50 ± 2.72	19.64	5.53 ± 0.39	1.53 ± 0.03	0.94 ± 0.19	0.69 ± 0.03
	150	23.22 ± 3.68	23.63 ± 2.0	11.37	5.20 ± 0.30	1.51 ± 0.03	0.89 ± 0.27	0.76 ± 0.02
	300	22.33 ±	24.1 ± 2.34	24.76	5.36 ± 0.59	1.56 ± 0.10	0.70 ± 0.10	0.79 ± 0.44
	PC	25.43 ± 1.64	25.25 ± 2.43	-0.82	5.19 ± 0.21	1.49 ± 0.06	0.68 ± 0.14	0.82 ± 0.05

NC = Negative control (distilled water) *, **, *** Significant at p < 0.05, p < 0.01 and 0.001 respectively

Table 4.20. Body weight changes and relative organ weight of mice orally administered 1:1 mixture of AgNPs and CuONPs following 14 and 28- day exposure periods

	Conc (mg/kg)	Initial body weight (g)	Final body weight (g)	% change in body weight	Relative organ weight (%)			
					Liver	Kidney	Spleen	Testis
14 days	NC	25.4 ± 3.98	26.10 ± 2.10	2.76	4.60 ± 0.20	1.51 ± 0.10	0.53 ± 0.07	0.76 ± 0.06
	18.75	22.00 ± 0.69	27.05 ± 1.84	22.95	4.80 ± 0.10	1.32 ± 0.07	0.49 ± 0.04	0.66 ± 0.04
	37.5	23.48 ± 1.94	24.05 ± 0.10	2.45	5.80 ± 0.39	1.58 ± 0.07	0.80 ± 0.17	0.67 ± 0.04
	75	25.78 ± 2.01	20.15 ± 2.91	-21.83	5.11 ± 0.30	1.55 ± 0.07	0.40 ± 0.06	0.97 ± 0.08
	150	25.98 ± 0.84	28.10 ± 1.48	8.18	5.74 ± 0.35	1.38 ± 0.10	0.59 ± 0.11	0.59 ± 0.03
	300	26.27 ± 0.47	26.33 ± 1.70	1.40	5.72 ± 0.13	1.63 ± 0.10	0.46 ± 0.14	0.66 ± 0.05
	PC	23.68 ± 0.73	23.05 ± 0.82	-2.64	4.92 ± 0.59	1.69 ± 0.08	0.81 ± 0.10	0.82 ± 0.10
28 days	NC	25.15 ± 0.99	28.08 ± 3.23	1.42	4.72 ± 0.44	1.46 ± 0.12	0.43 ± 0.04	0.66 ± 0.05
	18.75	22.33 ± 0.21	27.40 ± 1.56	22.67	7.01 ± 0.33**	1.57 ± 0.07	1.63 ± 0.27**	0.77 ± 0.19
	37.5	21.15 ± 0.24	24.15 ± 3.68	14.06	5.95 ± 0.84	1.48 ± 0.05	0.92 ± 0.20	0.74 ± 0.03
	75	22.35 ± 1.31	26.90 ± 2.45	20.22	4.86 ± 0.08	1.46 ± 0.04	0.61 ± 0.20	0.72 ± 0.03
	150	21.37 ± 1.26	25.27 ± 1.54	18.51	6.01 ± 0.06	1.78 ± 0.23	0.82 ± 0.10	0.74 ± 0.09
	300	27.50 ± 1.90	29.8 ± 0.10	8.91	5.26 ± 0.23	1.74 ± 0.10	0.70 ± 0.17	0.73 ± 0.01
	PC	25.43 ± 1.64	25.25 ± 2.43	-0.82	5.19 ± 0.21	1.49 ± 0.06	0.68 ± 0.14	0.82 ± 0.05

NC = Negative control (distilled water) *, **Significant at p < 0.05 and < 0.01 respectively

For the 1:1 mixture group, mice exhibited a significant increase in PCV, Hb (at 37.5, 75 and 300 mg/kg) and RBC (at 37.5 and 75 mg/kg bw) compared to the negative control. A concomitant non – significant decrease ($p > 0.05$) in MCHC and MCV and an increase in MCH was also induced by the 1:1 mixture at 14th day. However, at the 28 – day exposure period, the 1:1 mixture induced a significant decrease in PCV, Hb, MCHC (at 150 mg/kg) and RBC (at 75 and 150 mg/kg) compared to the control. Also, a significant decrease in MCH and increase in MCV was observed at 75 and 37.5 mg/kg respectively (Table 4.23).

Table 4.22 and 4.24 shows the WBC counts and differentials, and platelet in exposed mice at both 14 and 28 – exposure periods. At 14 days, AgNPs did not induce a significant change in WBC and its indices but there was a decrease ($p < 0.05$) in platelet counts at 37.5 mg/kg compared to the control. However, at 28 – day exposure period there was a significant increase ($p < 0.05$) in WBC at the 300 mg/kg concentration compared to the control.

For the CuONPs exposed mice at 14 – exposure period, a significant decrease ($p < 0.05$) in WBC and neutrophils was observed at 75 mg/kg compared to the negative control group. Similarly, at 28- day exposure, a significant decrease ($p < 0.05$) in WBC at 75 – 300 mg/kg bw; platelet count (at 150 and 300 mg/kg); increase in lymphocyte (at 18.75 and 150 mg/kg); and neutrophils (at 37.5 - 150 mg/kg) compared to the control were recorded (Table 4.22).

Furthermore, at 14 –day exposure period, the 1:1 mixture induced a significant decrease ($p < 0.05$) in WBC at 75 – 300 mg/kg compared to the control. Decreases in platelet count (at 150 and 300 mg/kg); neutrophils (at 18.75, 75, 150 mg/kg) compared to the control were also recorded. However, the 1:1 mixture induced increase in lymphocytes, as well as monocytes, compared to the NC. Similarly, at 28 days, the 1:1 mixture induced a significant decrease in WBC and platelet count at 75 – 300 mg/kg compared to the control. There was a concomitant significant increase ($p < 0.05$) in lymphocytes at all concentrations compared to the control (Table 4.24).

Table 4.21. Red blood cell (RBC) and Red cell indices of mice exposed to AgNPs and

Parameters	Conc. (mg/kg bw)	AgNPs		CuONPs	
		14 days	28 days	14 days	28
PCV	NC	43.00± 4.00	27.00 ± 1.00	43.00±4.00	27.00 ± 1.00
	18.75	27.14± 2.37	37.67 ±2.73*	37.33±3.17	29.67±3.38
	37.50	35.25 ±5.59	35.67±1.33	36.25±1.88	36.50±3.23
	75.00	41.67±4.91	42.00 ±2.86*	34.67±3.33	37.50±3.43
	150.00	38.00±0.70	35.50±1.04	43.50±2.02	34.50±1.66
	300.00	41.33±10.72	33.25 ±4.13	34.50±4.51	32.00±5.77
	PC	37.00±1.73	29.25±4.53	37.00±1.73	29.25±4.53
	Hb(g/dl)	NC	14.23±1.32	8.40 ± 0.20	14.23±1.31
18.75		8.87±.77	12.57 ±0.91*	12.40±1.31	9.90±1.25
37.50		11.28±1.89	12.43±.63*	12.05±0.64	11.80±1.16
75.00		13.93±1.74	14.25±0.99*	11.46±1.28	12.65±1.16*
150.00		13.38±0.32	11.93±0.23*	14.55±0.69	11.40±0.44
300.00		13.40±3.37	10.90±1.32	12.47±0.99	10.50±1.88
PC		12.50±0.61	9.45±1.48	12.50±0.60	9.45±1.48
RBC (x 10 ⁶)		NC	6.82±0.72	4.20 ±0.20	6.82±0.71
	18.75	4.27±0.39	6.29±0.61*	6.42±0.68	4.69±0.63
	37.50	5.53±1.09	5.98±0.34	5.90±0.33	5.80±0.60
	75.00	7.10±0.96	7.07±0.54*	5.62±0.71	6.24±0.68*
	150.00	6.44±0.10	5.86±0.23	7.19±0.10	5.37±0.36
	300.00	6.80±1.80	5.57±0.72	5.63±0.82	5.34±1.01
	PC	5.98±0.33	4.68±0.86	5.98±0.33	4.68±0.86
	MCHC (%)	NC	33.10±0.64	33.10±0.06	33.10±0.06
18.75		32.70±0.37	32.70±0.37	32.70±0.37	33.27±0.57
37.50		31.87±0.63	31.88±0.63	31.88±0.63*	32.25±0.79
75.00		33.38±0.33	33.38±0.33	33.38±0.33*	33.74±0.23
150.00		35.21±0.81	35.21±0.80	35.21±0.80	33.09±0.34
300.00		32.63±0.44	32.64±0.45	32.64±0.45	32.85±0.20
PC		33.78±0.14	33.78±0.14	33.78±0.14	32.29±0.36
MCH(pg)		NC	20.93±0.35	19.90 ±0.38	20.93±0.35
	18.75	20.86±0.48	20.10±1.00	20.86±0.48	21.18±0.61
	37.50	20.65±0.53	20.80±0.53	20.65±0.53	20.40±0.48
	75.00	19.67±0.25	20.22±0.30	19.67±0.24	20.43±0.52
	150.00	20.78±0.44	20.41±0.57	20.78±0.43	21.34±0.63
	300.00	19.97±0.65	19.68±0.45	19.97±0.65	19.78±0.31
	PC	20.92±0.53	20.48±0.67	20.92±0.53	20.48±0.67
	MCV(fl)	NC	63.21±0.98	63.97±2.07	63.21±0.98
18.75		63.79±1.41	60.17±1.99	58.43±1.23	63.67±1.74
37.50		64.86±2.06	59.75±1.25	61.57±1.50	63.29±.98
75.00		58.97±1.10	59.59±0.77	62.28±2.20	60.57±1.43
150.00		59.02±0.42	60.67±1.22	60.43±0.86	64.46±1.28
300.00		61.15±1.27	59.92±0.99	61.95±1.45	60.22±0.69
PC		61.93±1.37	63.50±2.60	61.93±1.37	63.50±2.60

Note : NC- Negative control (Distilled water) PC: Positive control cyclophosphamide * significant (p< 0.05) values are presented as mean ±SE(standard error of mean)

CuONPs for 14 and 28 days

Parameters	Conc (mg/L)	AgNPs		CuONPs	
		14 days	28 days	14 days	28 days
WBC (x 10 ³)	NC	5.33±0.36	5.60±0.98	5.33±0.36	5.60±0.98
	18.75	6.01±0.82	7.77±0.97	5.28±0.36	8.88± 0.82
	37.50	8.35±0.14	6.27±0.62	6.00±0.40	7.56± 0.77
	75.00	6.05±0.76	8.61±0.84	4.33±0.39*	6.26± 0.10
	150.00	7.21±0.93	7.94±0.15	4.04±0.88	7.70±0.88
	300.00	8.93±0.97	9.56±0.66*	7.78±0.36	6.76± 0.56
	PC	4.83±0.60	5.24±0.90	4.83±0.60*	5.24±0.90
PLATELET (x10 ⁶)	NC	0.14±0.03	0.18±0.02	0.14±0.03	0.18±0.02
	18.75	0.12±0.09	0.11±0.06	0.35±0.02	0.15±0.22
	37.50	0.19±0.03	0.11±0.06	0.10±0.01	0.15±0.36
	75.00	0.11±0.01	0.12±0.06	0.80±0.01	0.12±0.02
	150.00	0.13±0.06	0.16±0.03	0.88±0.01	0.16±0.02
	300.00	0.14±0.01	0.19±0.03	0.15±0.07	0.12±0.01
	PC	0.12±0.05	0.14±0.03	0.12±0.05	0.14±0.03
LYMPH	NC	58.00±2.51	69.50 ± 2.50	51.80±6.61	44.60± 1.02
	18.75	64.14±2.96	67.00±4.36	60.00±4.95	62.40± 3.56
	37.50	62.25±1.65	65.00±1.53	54.60±6.02	64.00±4.71
	75.00	71.00±1.15	70.75±2.66	64.60±4.82	69.00± 2.07
	150.00	64.50±2.02	69.75±1.65	44.33±6.66	66.20± 2.96
	300.00	55.67±5.89	65.00±3.11	59.40±9.86	60.60± 3.12
	PC	67.33±2.03	68.25±3.50	54.60±11.30	53.60± 2.32
NEUTERO PHILS	NC	37.67±2.33	28.25±3.59	41.40±7.09	46.40 ± 0.93
	18.75	31.29±2.89	29.67±4.05	33.80± 3.70	30.20 ±3.81
	37.50	33.50±1.44	31.00±1.53	37.80± 6.61	29.60 ± 1.63
	75.00	26.33±2.18	25.75±3.30	28.00±4.90*	23.40 ± 2.16
	150.00	31.75±2.95	26.75±1.88	46.00±6.24	27.00 ± 3.08
	300.00	40.33±5.81	32.25±2.95	34.60±9.07	31.80 ± 3.95
	PC	29.00±1.15	25.00±1.58	37.80±11.19*	39.60 ± 2.71
MONOCYT ES	NC	2.33±0.67	2.00±0.41	2.20±1.30	3.40 ± 0.68
	18.75	2.71±0.18	2.00±0.58	3.20±1.10	4.60 ± 0.24
	37.50	2.00±0.41	2.00±0.58	4.40±1.14	3.00 ± 0.71
	75.00	1.67±0.67	1.75±0.48	4.20±0.84	3.20 ± 0.37
	150.00	2.00±0.41	1.75±0.47	4.33±0.58	2.80 ± 0.73
	300.00	1.67±0.33	1.75±0.47	3.80±1.10	4.00 ± 0.45
	PC	1.67±0.33	1.75±0.47	3.80±1.92	2.60 ± 0.50
EOSINOPH ILS	NC	2.00±0.57	1.85±0.16	4.40±0.55	5.20 ± 0.58
	18.75	1.85±0.26	1.75±0.75	2.60±1.34	2.40 ± 0.93
	37.50	2.25±0.48	1.33±0.67	3.00±1.58	3.20 ± 0.80
	75.00	1.00±0.57	2.00±0.00	3.20±0.45	3.80 ± 0.49
	150.00	1.75±0.63	1.75±0.47	5.00±1.00	3.60 ± 1.17

	300.00	2.33±1.20	1.75±0.25	1.80±1.30*	3.40 ± 0.81
PC		2.00±1.00	1.00±0.41	3.20±1.30	3.80 ± 0.73

Table 4.22. White blood cells count and differentials of mice exposed to AgNPs and CuONPs for 14 and 28 days
 NB: NC: Negative control (Distilled water) PC: Positive control cyclophosphamide *Significant ($p < 0.05$) values are presented as mean ±SE (standard error of mean)

Table 4.23. Red blood cell (RBC) indices and Red cell indices of mice exposed to 1:1 mixture of AgNPs and CuONPs for 14 and 28 days

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Parameters	Conc. (mg/Kg bw)	Exposure periods (Mean ± SE)	
		14 days	28 days
PCV	NC	34.00 ± 2.88	35.80± 2.08
	18.75	27.40 ± 2.46	23.80± 1.85
	37.50	20.40 ± 1.72*	20.80± 1.16
	75.00	23.00 ± 1.41*	26.80± 1.07
	150.00	28.80 ± 2.56	24.00± 1.05*
	300.00	24.20 ± 1.36*	21.50± 1.44
	PC	24.60 ± 3.61	31.20± 3.61
	Hb(g/dl)	NC	11.32 ±0.98
18.75		9.24 ±.84	8.08± 0.69
37.50		6.76 ±.64*	6.80± 0.29
75.00		7.62 ±.39*	8.84± 0.25
150.00		9.14 ±.87	7.98± 0.44*
300.00		7.76 ±.39*	7.25± 0.43
PC		7.98 ±1.62	10.34± 1.34
RBC (x 10 ⁶)		NC	3.55 ± 0.21
	18.75	3.29 ± 0.37	2.24± 0.33
	37.50	1.98 ± 0.44*	1.63± 0.05
	75.00	2.00 ± 0.36*	3.34± 0.25*
	150.00	3.01 ± 0.37	2.25± 0.43*
	300.00	2.29 ± 0.37	1.94± 0.27
	PC	2.43 ± 0.35	3.43± 0.35
	MCHC (%)	NC	33.27 ± 0.244
18.75		33.70 ± 0.14	33.89± 0.53
37.50		33.08 ± 0.78	32.80± 0.57
75.00		33.24± 0.76	33.08± 0.78
150.00		31.74 ± 0.68	33.20± 0.49*
300.00		32.12 ± 0.43	33.78± 0.34
PC		32.54 ± 1.30	32.99± 0.72
MCH(pg)		NC	31.84 ± 1.51
	18.75	28.58 ±1.34	37.51± 2.72
	37.50	36.86 ±3.33	42.09± 2.84
	75.00	40.90 ±4.62	27.02± 1.79*
	150.00	31.71 ±3.25	38.83± 4.33
	300.00	36.54 ±4.16	38.44± 2.94
	PC	35.34±7.69	30.26± 2.22
	MCV(fl)	NC	95.66 ± 4.31
18.75		84.88 ± 4.32	110.66± 7.65
37.50		112.05 ± 11.15	129.01± 10.71*
75.00		122.23 ± 11.86	81.38± 3.65
150.00		99.37 ± 8.43	117.61± 14.08
300.00		113.67 ± 12.73	113.73± 8.47
PC		108.33 ± 21.86	91.42± 5.34

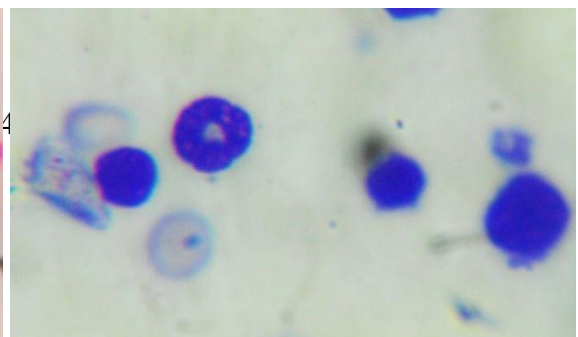
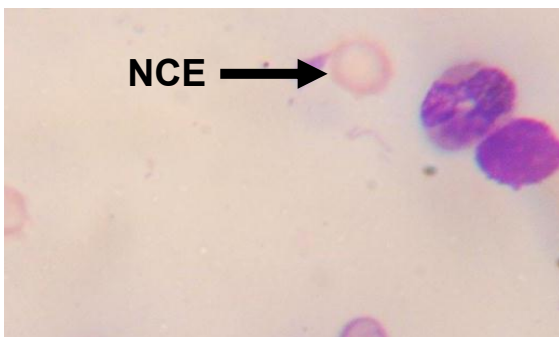
NC- Negative control (Distilled water) PC: Positive - control cyclophosphamide

*Significant (p< 0.05) values are presented as mean ±SE(standard error of mean)

Table 4.24. White blood cells count and differentials of mice exposed to mixture of AgNPs and CuONPs for 14 and 28 days

Parameters	Concentration (mg/L)	Exposure periods (Mean ± SE)	
		14 days	28 days
WBC	NC	32270 ± 3606	24190± 2966
	18.75	23800 ±3290	16430± 2015
	37.50	25290±2388	18900± 1778
	75.00	17130±1813*	16540± 2482*
	150.00	15090±2236*	17160± 1204*
	300.00	15360±1759*	14963± 1740*
	PC	19420 ± 8175	24550± 1113
	PLATELET	NC	283200 ± 52602
18.75		278400±44841	208800± 32455
37.50		322200±26147	213000± 19478
75.00		183600±21720	235800± 31813*
150.00		151200±20691*	287800± 24287*
300.00		137000±8282*	248000± 52942*
PC		271400 ± 99014	259200± 27029
LYMPH		NC	51.80 ± 2.96
	18.75	62.40 ±1.66*	56.80± 3.98*
	37.50	55.20 ±1.59	49.80± 1.88*
	75.00	59.40 ±2.38	53.00± 2.07*
	150.00	61.80 ±2.65*	52.60± 1.91*
	300.00	59.60 ±2.69	58.75 ± 2.02*
	PC	54.60 ± 5.60	53.60± 2.32
	HETEROPHILS	NC	41.40 ± 3.17
18.75		31.00 ±1.87*	36.20± 3.87
37.50		38.60 ±1.50	42.40± 1.78
75.00		30.80 ±2.00*	45.40± 4.50
150.00		31.00 ±3.21*	47.40± 5.11
300.00		32.40 ±2.58	32.50± 1.44
PC		37.80 ± 5.00	39.60± 2.71
MONOCYTES		NC	2.20 ± 0.58
	18.75	3.00 ± 0.71	3.20± 0.58
	37.50	2.20 ± 0.73	3.40± 0.81
	75.00	3.80 ± 0.37	3.20± 0.66
	150.00	2.80 ± 0.66	3.80± 0.97
	300.00	4.60 ± 0.51*	4.50± 0.29
	PC	3.80 ± 1.92	2.60± 0.51
	EOSINOPHILS	NC	4.40 ± 0.24
18.75		3.60 ± 0.75	3.60± 1.03
37.50		3.80 ± 0.86	4.00± 0.89
75.00		3.40 ± 0.40	4.00± 0.89
150.00		3.20 ± 0.86	4.00± 0.63
300.00		3.00 ± 0.71	4.00± 0.41
PC		3.20 ± 1.30	3.80± 0.73

NC- Negative control (Distilled water) PC: Positive - control cyclophosphamide *Significant (p< 0.05)



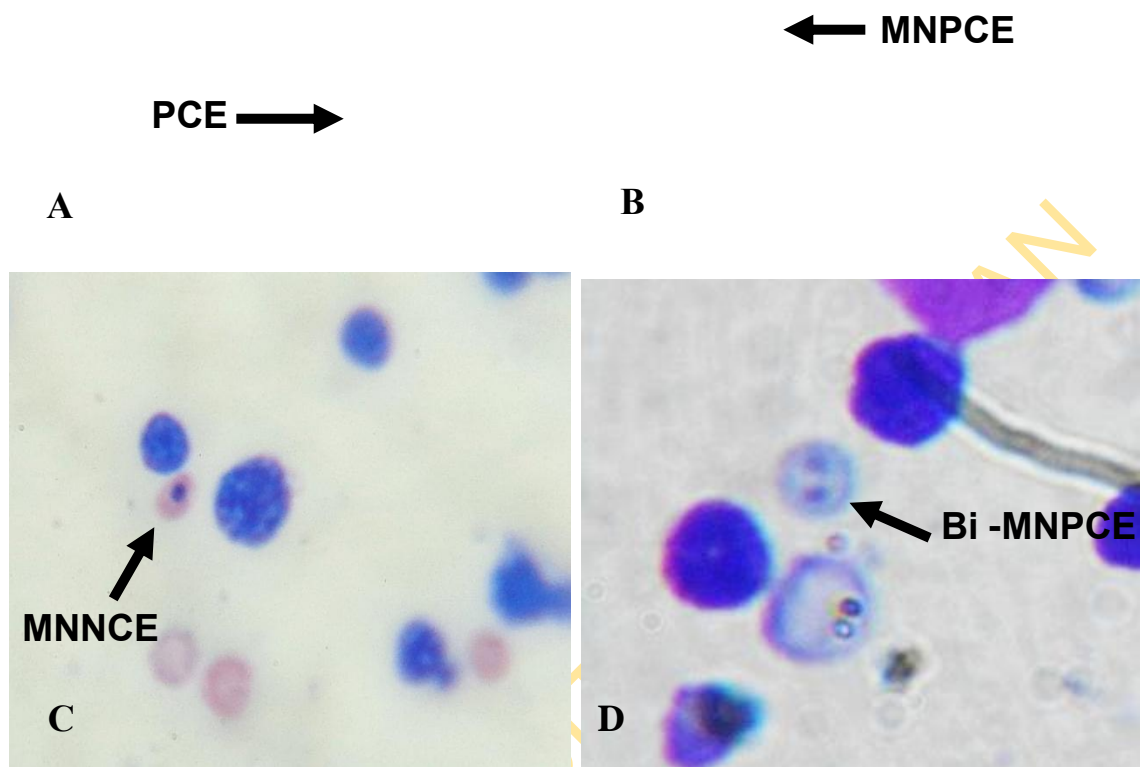


Figure 4.32. Micronuclei induced in bone marrow of mice exposed to silver and copper(ii) oxide nanoparticles, and their mixture in mice (X 1000):

- (A) Polychromatic erythrocytes (PCEs) and Normochromatic erythrocytes (NCE)
- (B) Micronucleated polychromatic erythrocyte (MNPCE)
- (C) Micronucleated normochromatic erythrocyte (MNNCE)
- (D) Bi – micronucleated polychromatic erythrocyte (Bi – MNPCE)

4.4.5 Histological alterations in liver, kidney, spleen and testes of mice exposed to AgNPs, CuONPs and their 1:1 mixture

The histological alterations observed in liver, kidney, spleen and testis of mice exposed to silver and copper(II) oxide nanoparticles, and their 1:1 mixture for 14 and 28 – day exposure periods are presented in Figures 4.34 – 4.41. The hepatocytes of mice in the negative control were closely packed with distinct nuclei. The portal triad and the central vein are intact. However, there were vacuolar degenerations in the hepatocytes, kupffer cell hyperplasia and necrosis in the liver of mice exposed to AgNPs, CuONPs and the 1:1 mixture (Figures 4.34 and 4.35). The kidney of mice in the unexposed group did not present any visible lesion but histological alterations such as tubular epithelial degeneration, protein casts and necrosis were observed in the nephrons of exposed mice (Figures 4.36 and 4.37). Spleen of mice exposed to AgNPs, CuONPs and their 1:1 mixture revealed marked follicular lymphoid hyperplasia and depletion, and widely space Periarteriolar Lymphoid Sheaths (PALS) with marked congestion of splenic sinuses and sinusoids are some of the observed alterations in the spleen of exposed mice (Figures 4.38 and 4.39). The testes of mice in the negative control group showed normal architectural structure of the testis with presence of numerous spermatogenic cells while testicular lesions like degeneration and loss of basal cells, loss of spermatogenic cells, hypospermia as well as reduced germinal epithelium, necrosis of spermatogenic cells (Figures 4.40 – 4.41) were recorded in the exposed animals..

4.4.6 Hepatic oxidative stress biomarkers in mice

Tables 4.25 – 4.27 show the effect of AgNPs, CuONPs and their 1:1 mixture on the activities of antioxidant enzymes/molecules in the liver of mice exposed for 14 and 28 days. At 14 days, AgNPs induced significant increase ($p < 0.05$) in levels of MDA (at 75 and 150 mg/kg) and a concomitant decrease in GSH, SOD and CAT activity. At the 28- exposure period, AgNPs also induced an increase in MDA; a significant increase in CAT (at 150 mg/kg); a significant decrease in GSH (at 18.7, 37.5, 150 and 300 mg/kg) and a decrease in levels of SOD across the concentrations (Table 4.25).

At 14 – day exposure to CuONPs, levels of MDA decreased at all the concentrations with concomitant increase in GSH and depletion of SOD and CAT (at 37.5 and 75.00

mg/kg). Within the recovery period at the 28th day, levels of MDA increased while GSH and SOD decreased concomitantly and CAT production increased (Table 4.26).

At the 14 – day exposure, 1:1 mixture of AgNPs and CuONPs induced a significant increase in levels of MDA (at 37.5, 75 and 300 mg/kg). The hepatic levels of GSH, SOD increased and CAT was significantly decreased across all concentrations. Moreover, at 28 – day exposure, GSH, and SOD levels decreased while CAT production was also increased concomitantly with increase in MDA (Table 4.27).

4.4.7 Serum markers of hepatic and renal injury

The effect of AgNPs, CuONPs and their 1:1 mixture on Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) activities are presented in Tables 4.28 – 4.30. during the 14 – day exposure period, AgNPs significantly increased the levels of ALT (at 18.75, 37.5 and 150 mg/kg); AST (18.75, 75.00 and 150.00 mg/kg) and Urea at all concentrations. At 28 - day exposure period, the hepatic and renal injury heightened with significant ($p < 0.05$) decrease in ALT (at 75.00 and 300.00 mg/kg) and concomitant increase in AST as well as significant ($p < 0.05$) increase Urea levels (at 18.75, 37.50, 75.00 and 300.00 mg/kg) (Table 4.28).

The CuONPs and the 1:1 mixture induced similar patterns of alterations in the activities of ALT, AST and Urea concentrations at both 14 and 28 – day exposure periods. CuONPs significantly ($p < 0.05$) increased the ALT and Urea levels at across all concentrations as well as AST (at 18.75, 37.50 and 75.00 mg/kg). At the 28th day, CuONPs induced a decrease in ALT and an increase in AST and a concomitant significant increase in Urea concentrations at 75.00 and 300. 00 mg/kg (Table 4.29).

At 14 – day exposure period, the 1:1 mixture induced significant ($p < 0.05$) increases in the activities of ALT (at 18.75, 37.50, 75.00 and 150.00 mg/kg), AST (at 37.50, 75.00 and 150.00 mg/kg) and Urea (at 18.75, 37.50, 75.00 and 150.00 mg/kg). The activities of ALT, AST and Urea further increased at the 28 – day exposure period. The nanoparticle 1:1 mixture induced decrease in ALT level and significantly increase ($p < 0.05$) in the level of AST (at 18.75 mg/kg) and Urea (at 75 mg/kg – 300 mg/kg) (Table 4.30).

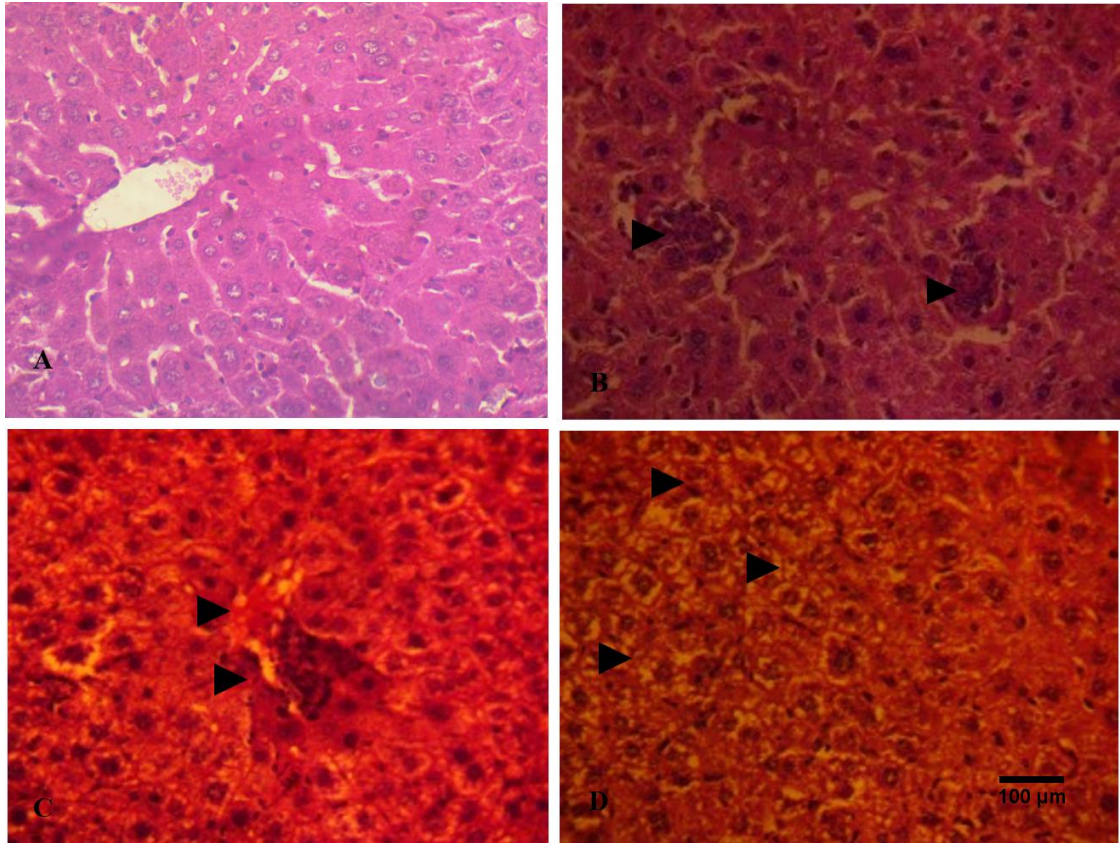


Figure 4. 34. Histological changes in liver of mice exposed to silver and copper(II) oxide nanoparticles, and their 1:1 mixture following 14 days exposure (H and E; x 400)

(A) Negative control: No visible lesion

(B) Diffuse degeneration and necrosis of hepatocytes (blue arrow) with foci of inflammatory cells (black arrow) at 75 mg/kg bw AgNPs

(C) Multifocal necrosis at 75 mg/kg bw CuONPs

(D) Centrilobular degeneration and necrosis of hepatocytes at 37.5 mg/kg bw of the 1:1 mixture (1:1)

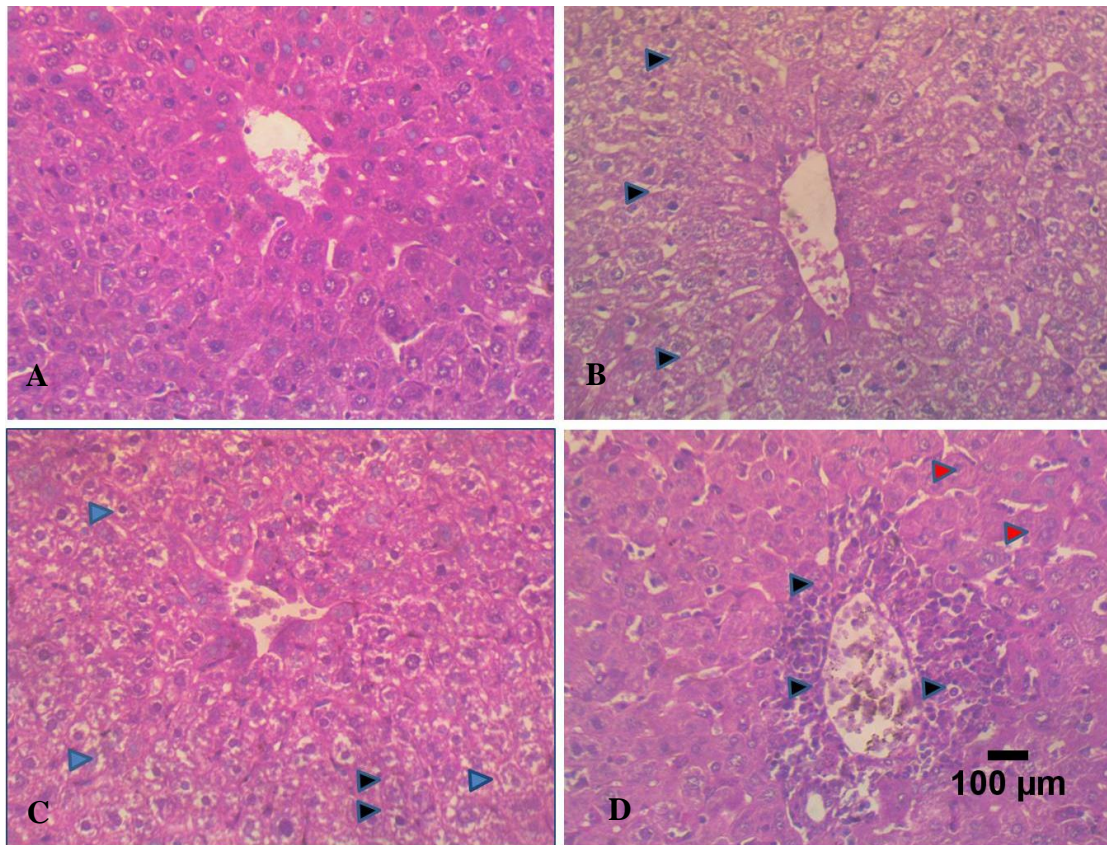


Figure 4.35. Histological changes in liver of mice exposed to silver and copper(II) oxide nanoparticles and their 1:1 mixture following 28 days exposure (H and E; x 400)

(A) Negative control: No visible lesion

(B) Moderate vacuolar degeneration of hepatocytes and mild mononuclear cellular infiltration at 300 mg/kg bw AgNPs

(C) Hepatocellular necrosis (blue arrow head) and dense mononuclear cellular infiltration (black arrow head) at 150 mg/kg bw CuONPs

(D) Single-cell necrosis of hepatocytes (black arrow head) and dense aggregates of mononuclear cells at the portal tracts (red arrow head) at 18.75 mg/kg bw of the 1:1 mixture

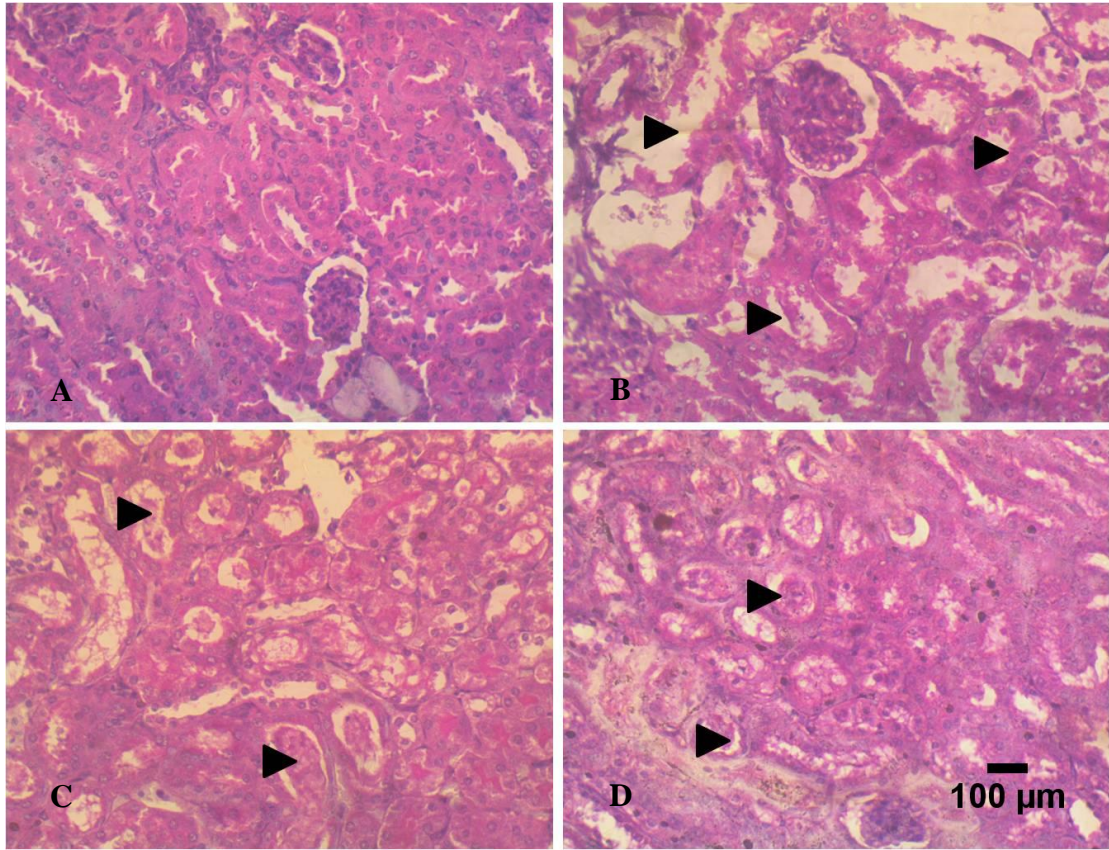


Figure 4.36. Histological changes in kidney of mice exposed to silver and copper(II) oxide nanoparticles and their 1:1 mixture following 14 days exposure (H and E; x 400)

(A) Negative Control

(B) Diffuse severe tubular epithelial degeneration and necrosis at 75 mg/kg bw AgNPs

(C) Tubular epithelial degeneration and necrosis at 75 mg/kg bw CuONPs

(D) Tubular epithelial degeneration with protein cast in the lumen and necrosis at 37.5 mg/kg bw 1:1 Mixture

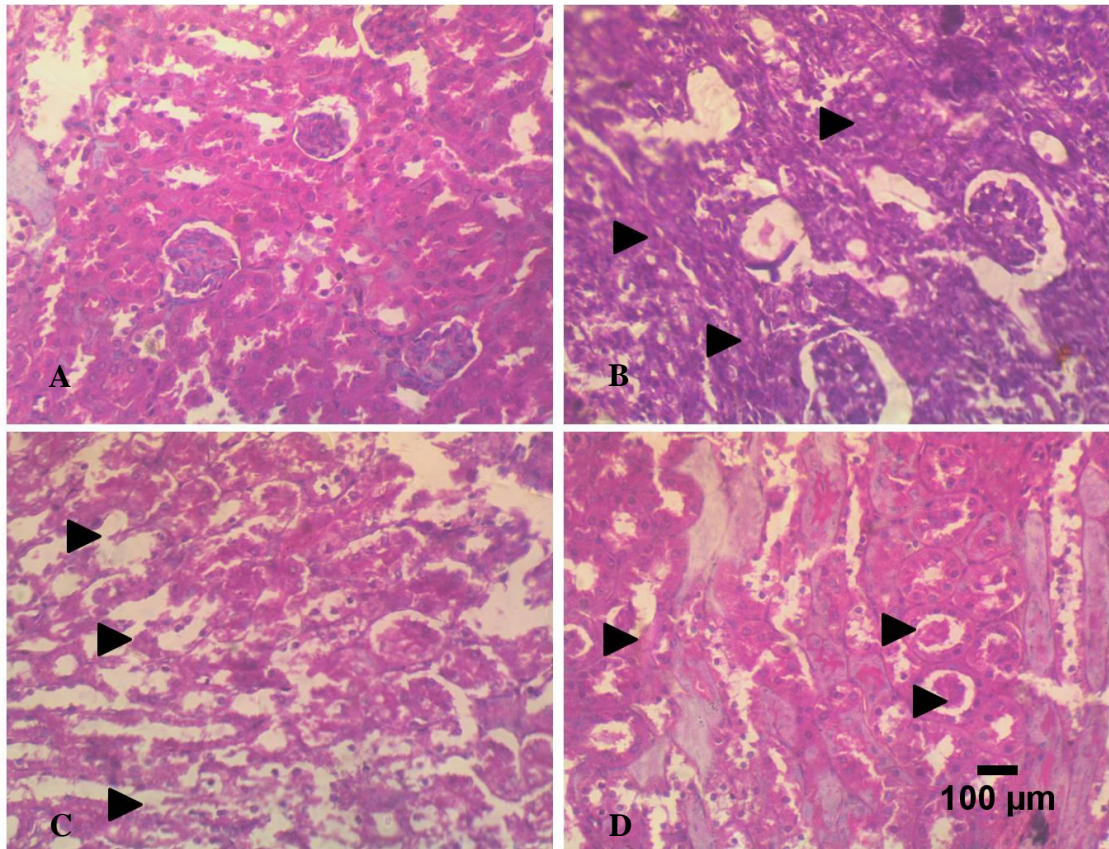


Figure 4.37. Histological changes in kidney of mice exposed to silver and copper(II) oxide nanoparticles and their 1:1 mixture following 28 - day exposure (H and E; x 400)

(A) Negative Control

(B) Neoplastic mass causing compressional atrophy on the adjacent renal parenchyma, tubular degeneration and necrosis at 37.5 mg/kg bw AgNPs

(C) Tubules show sloughing off of tubular epithelium, tubular degeneration and necrosis at 37.5 mg/kg bw CuONPs

(D) Multiple foci of sloughing off of tubular epithelial cells with the accumulation of intraluminal tubular casts at 37.5 mg/kg bw 1:1 Mixture

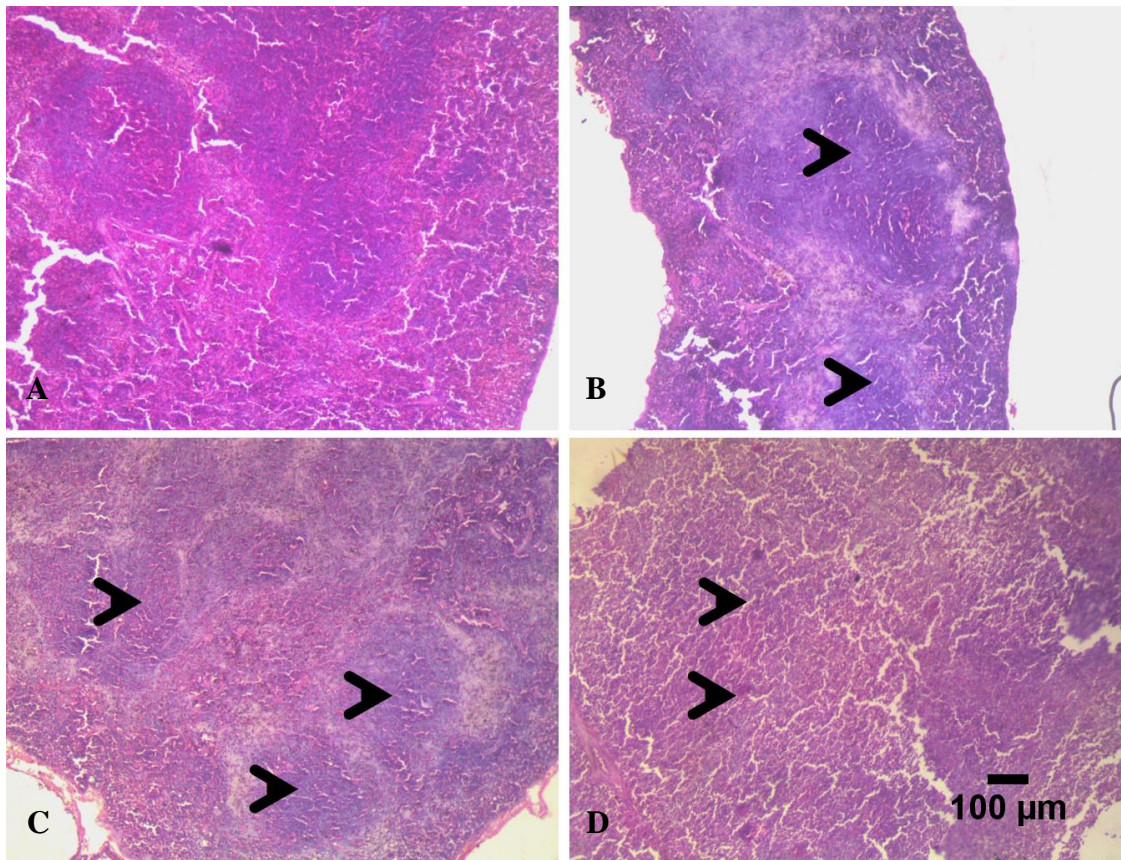


Figure 4.38. Histological changes in spleen of mice exposed to silver and copper(ii) oxide nanoparticles and their 1:1 mixture following 14 days exposure (H and E; x 400)

(A) Negative Control

(B) moderate follicular lymphoid hyperplasia at 300 mg/kg bw AgNPs

(C) marked follicular lymphoid hyperplasia at 75 mg/kg bw CuONPs

(D) mild follicular lymphoid depletion at 18.75 mg/kg bw CuONPs

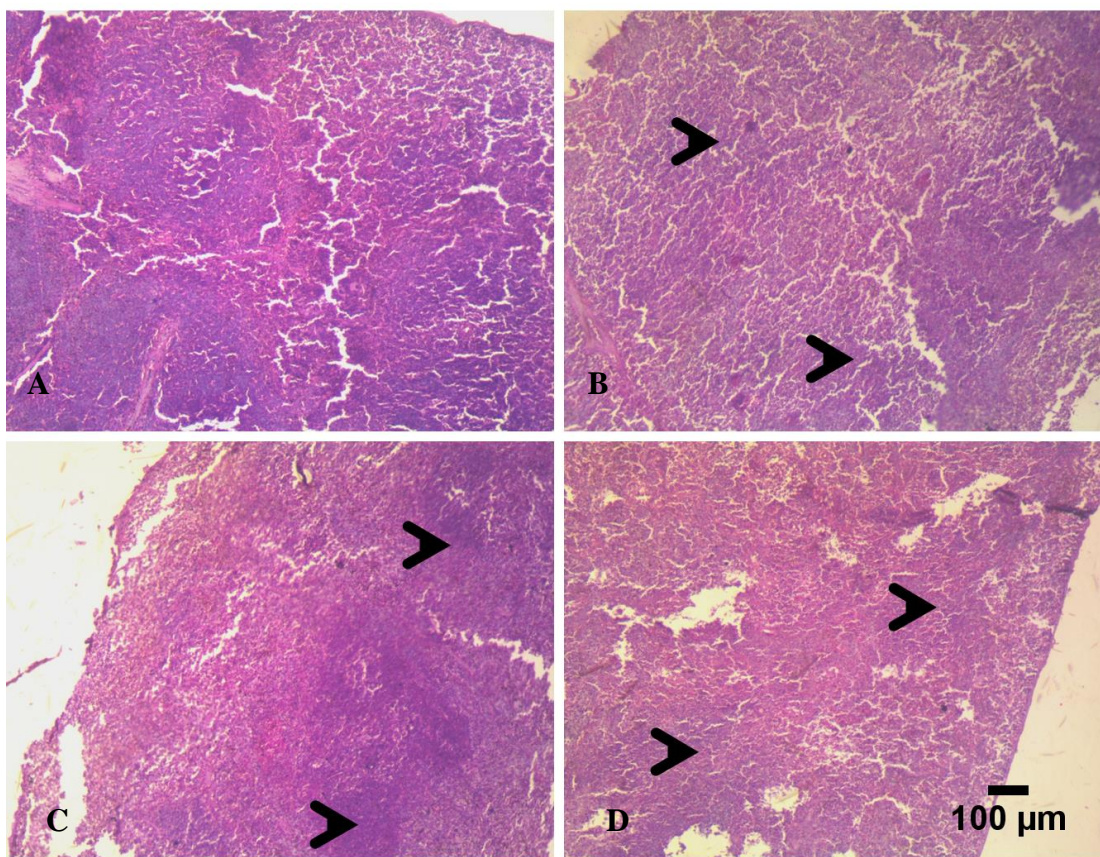


Figure 4. 39. Histological changes in spleen of mice exposed to silver and copper(ii) oxide nanoparticles and their 1:1 mixture following 28 - day exposure period (H and E; x 400).

(A) Negative Control

(B) Few widely-spaced distinct PALSs/lymphoid follicles, marked congestion of the splenic sinuses and sinusoids and multiple foci of haemosiderin-laden macrophages at 150 mg/kg bw AgNPs

(C) Moderate numbers of discrete widely-spaced large PALS at 150 mg/kg CuONPs

(D) Paucity of distinct PALSs/lymphoid follicles, marked congestion of the splenic sinuses and sinusoids, multiple foci of haemosiderin-laden macrophages at 150 mg/kg Mixture (1:1)

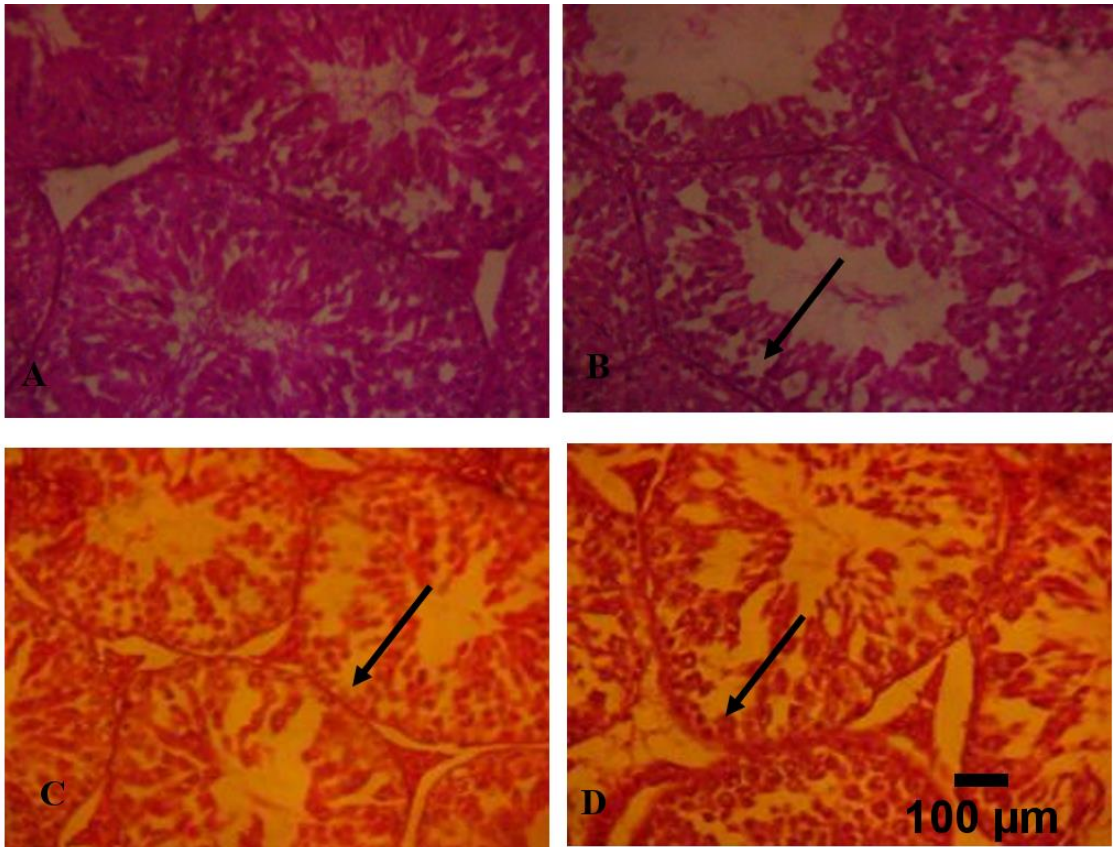


Figure 4.40. Histological changes in testis of mice exposed to silver and copper(ii) oxide nanoparticles and their 1:1 mixture following 14 days exposure (H and E; x 400).

(A) Negative Control – no visible lesion

(B) Testicular degeneration and loss of basal cells at 150 mg/kg bw of AgNPs

(C) Marked testicular degeneration, and loss of spermatogenic cells at 18.75 mg/kg bw of CuONPs

(D) Loss of spermatogenic cells at 18.75 mg.kg bw of the 1:1 mixture

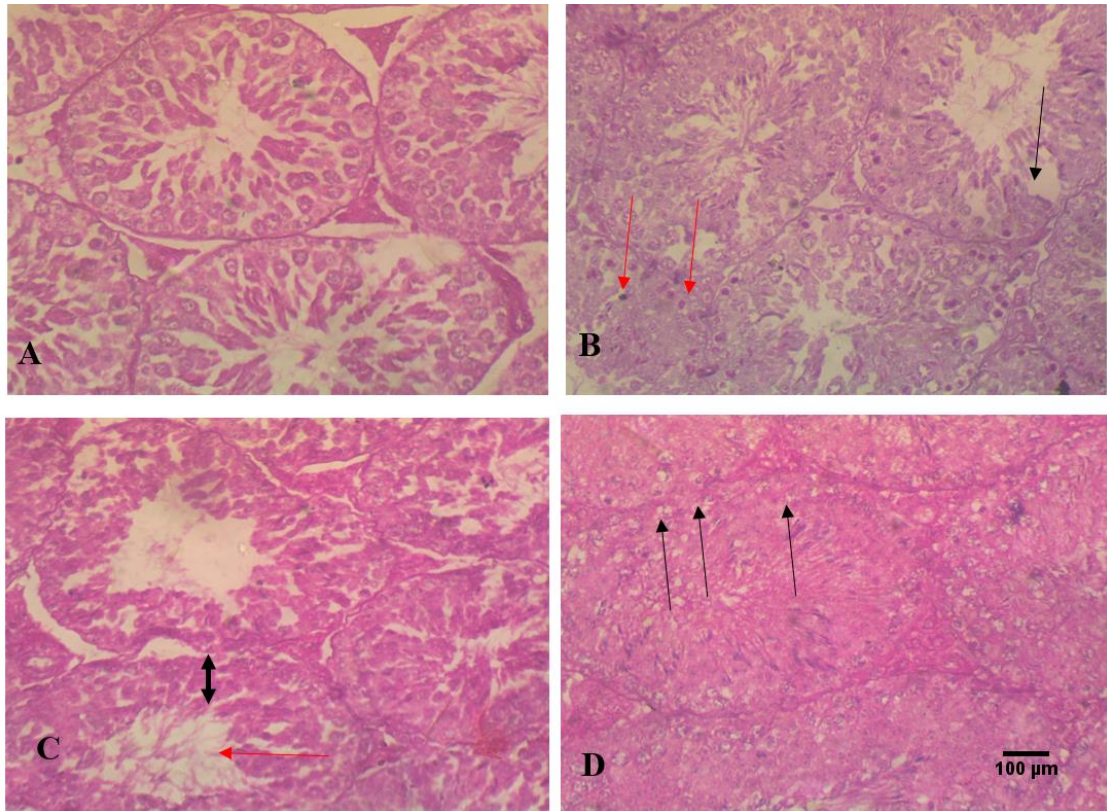


Figure 4.41. Histological changes in testis of mice exposed to silver and copper(ii) oxide nanoparticles and their mixture following 28 days exposure (H and E; x 400)

(A) Negative Control

(B) Moderate depletion of spermatogenic cells (red arrow), multiple foci of darkly-staining degenerate and necrotic spermatogenic cells (black arrow) at the basal compartment at 150 mg/kg bw AgNPs

(C) Moderately depletion of spermatogenic cells (red arrow). The height of the germinal epithelium is reduced and the lumen consequently widened (black arrow) at 150 mg/kg bw CuONPs

(D) Few foci of seminiferous tubules that show mild vacuolar change/degeneration of a few basal germinal epithelial cells at mg/kg bw NPs mixture

Table 4.25. Effects of AgNPs on the levels of oxidative stress biomarkers in mice following 14 and 28- days exposure periods

Concentrations (mg/L)	MDA (unit/mg protein) x 10 ⁻⁶		GSH (unit/mg protein)		SOD (unit/mg protein)		CAT (μmol H ₂ O ₂ consumed/min/mg protein)	
	14 day	28 days	14 day	28 days	14 day	28 days	14 day	28 days
NC	0.16 ± 0.01	0.16 ± 0.01	17.04 ± 0.52	28.15 ± 0.53	1.56 ± 0.11	2.33 ± 0.00	217 ± 9.13	218 ± 11.30
18.75	0.15 ± 0.01	0.22 ± 0.03	16.46 ± 0.30	25.56 ± 0.28*	1.33 ± 0.00	1.17 ± 0.17	154 ± 11.2***	182 ± 14.40
37.50	0.18 ± 0.002	0.18 ± 0.01	14.35 ± 0.93*	24.63 ± 0.32*	1.44 ± 0.11	1.33 ± 0.00	179 ± 8.70*	258 ± 8.64
75.00	0.20 ± 0.01*	0.19 ± 0.09	15.14 ± 0.54	25.95 ± 0.58	1.56 ± 0.11	2.17 ± 0.17	182 ± 20.00	242 ± 11.50
150.00	0.20 ± 0.01*	0.19 ± 0.00	15.12 ± 0.17	24.83 ± 0.49*	1.17 ± 0.17	0.50 ± 0.17	111 ± 4.57***	269 ± 5.46*
300.00	0.19 ± 0.01	0.19 ± 0.01	16.36 ± 0.50	26.48 ± 1.08*	1.00 ± 0.00	1.67 ± 0.33	259 ± 7.17*	247 ± 13.50
PC	0.18 ± 0.00	0.17 ± 0.01	19.82 ± 0.31*	24.52 ± 1.23*	1.17 ± 0.17	3.00 ± 0.00	78.8 ± 6.44***	226 ± 25.60

NC = Negative control (distilled water) PC = Positive control (cyclophosphamide 20 mg/kg *, **, *** Significant at p < 0.05, p < 0.01 and 0.001 respectively

Conc. (mg/L)	MDA (unit/mg protein)		GSH (unit/mg protein)		SOD(unit/mg protein)		CAT($\mu\text{mol H}_2\text{O}_2$ consumed/min/mg protein)	
	14 day	28 days	14 day	28 days	14 day	28 days	14 day	28 days
NC	0.16 \pm 0.01	0.16 \pm 0.01	17.04 \pm 0.52	28.15 \pm 0.53	1.56 \pm 0.11	2.33 \pm 0.00	217 \pm 9.13	218 \pm 11.30
18.75	0.083 \pm 0.01***	0.21 \pm 0.02	17.28 \pm 0.86	26.06 \pm 0.20	1.56 \pm 0.22	2.00 \pm 0.00*	236 \pm 1.37	201 \pm 4.40
37.50	0.14 \pm 0.00	0.20 \pm 0.02	17.37 \pm 0.53*	27.29 \pm 0.55	1.33 \pm 0.33	1.33 \pm 0.33 *	200 \pm 12.00	246 \pm 13.00
75.00	0.14 \pm 0.00	0.19 \pm 0.01	16.98 \pm 0.09	27.48 \pm 0.60	1.50 \pm 0.17	1.56 \pm 0.11	199 \pm 10.10	222 \pm 18.00
150.00	0.13 \pm 0.01	0.21 \pm 0.04	17.70 \pm 0.16	23.47 \pm 0.24**	1.33 \pm 0.00	2.56 \pm 0.22	222 \pm 17.40	259 \pm 14.90
300.00	0.14 \pm 0.004	0.18 \pm 0.02	19.69 \pm 0.19	27.54 \pm 0.72	1.33 \pm 0.33	1.50 \pm 0.17	227 \pm 9.35	234 \pm 2.89
PC	0.18 \pm 0.00	0.17 \pm 0.01	19.82 \pm 0.31*	24.52 \pm 1.23	1.17 \pm 0.17	3.00 \pm 0.00	78.8 \pm 6.44***	226 \pm 25.60

Table 4. 26. Effects of CuONPs on the levels of oxidative stress biomarkers in mice following 14 and 28 - day exposure periods

NC = Negative control (distilled water) PC = positive control (cyclophosphamide - 20 mg/kg) *, **, *** Significant at $p < 0.05$, $p < 0.01$ and 0.001 respectively

Table 4.27. Effects of 1:1 mixture of AgNPs and CuONPs on the levels of oxidative stress biomarkers in mice following 14 and 28- day exposure periods

Concentrations (mg/L)	MDA (unit/mg protein)		GSH (unit/mg protein)		SOD (unit/mg protein)		CAT ($\mu\text{mol H}_2\text{O}_2$ consumed/min/mg protein)	
	14 day	28 days	14 day	28 days	14 day	28 days	14 day	28 days
NC	0.16 \pm 0.01	0.16 \pm 0.01	17.04 \pm 0.52	28.15 \pm 0.53	1.56 \pm 0.11	2.33 \pm 0.00	217 \pm 9.13**	218 \pm 11.3
18.75	0.19 \pm 0.01	0.25 \pm 0.00	23.78 \pm 1.03	30.81 \pm 1.09	2.00 \pm 0.24	1.83 \pm 0.17	137 \pm 2.23**	318 \pm 6.34
37.50	0.23 \pm 0.02**	0.19 \pm 0.03	25.01 \pm 0.69	27.12 \pm 0.62	2.33 \pm 0.19*	2.50 \pm 0.71	71 \pm 21.20**	224 \pm 10.70
75.00	0.21 \pm 0.007*	0.23 \pm 0.02	24.02 \pm 0.95	25.97 \pm 0.57	2.22 \pm 0.11	1.67 \pm 0.67	46.6 \pm 6.47**	283 \pm 2.96
150.00	0.19 \pm 0.007	0.19 \pm 0.01	23.76 \pm 1.15	27.42 \pm 0.36	2.50 \pm 0.17*	0.67 \pm 0.22 *	129 \pm 2.79***	259 \pm 2.80
300.00	0.24 \pm 0.01**	0.17 \pm 0.01	27.18 \pm 0.57	29.20 \pm 0.57	2.22 \pm 0.11	1.22 \pm 0.22	133 \pm 25.4***	273 \pm 3.06
PC	0.18 \pm 0.00	0.17 \pm 0.01	19.82 \pm 0.31*	24.52 \pm 1.23	1.17 \pm 0.17	3.00 \pm 0.00	78.8 \pm 6.44***	226 \pm 25.6

NC = Negative control (distilled water) PC = Positive control (cyclophosphamide - 20 mg/kg) *, **, *** Significant at $p < 0.05$, $p < 0.01$ and 0.001 respectively

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Table 4.28. Effects of AgNPs on serum markers of hepatic and renal injury in mice following 14 and 28- day exposure periods

Concentration (mg/kg)	ALT (U/I)		AST (U/I)		Urea (mg/dL)	
	14 days	28 days	14 days	28 days	14 days	28 days
NC	13.2 ± 0.67	19.32 ± 0.27	42.1 ± 1.88	51.00 ± 0.50	122 ± 12.4	180 ± 2.59
18.75	15.70 ± 0.44*	19.29 ± 0.76	50.50 ± 1.95*	50.00 ± 6.00	173 ± 6.55*	258 ± 21.70**
37.50	16.00 ± 0.43*	18.14 ± 0.99	48.10 ± 1.88	55.50 ± 2.50	198 ± 3.10**	283 ± 4.14 ***
75.00	14.40 ± 0.14	15.78 ± 0.65*	52.60 ± 0.38*	58.5 ± 1.00	186 ± 6.10 **	363 ± 3.10***
150.00	16.2 ± 0.65*	18.57 ± 3.00	50.00 ± 1.30*	56.00 ± 3.00	199 ± 2.07 **	230 ± 26.9
300.00	-	14.70 ± 0.35**	-	56.50 ± 2.18	-	308 ± 3.16***
PC	12.3 ± 0.65	18.46 ± 0.54	44.8 ± 0.75	55.60 ± 0.89	226 ± 18.6***	3552.74***

NC = Negative control (distilled water) PC = positive control (cyclophosphamide - 20 mg/kg) *, **, *** Significant at p < 0.05, p < 0.01 and 0.001 respectively

- Lost samples

Table 4.29. Effects of CuONPs on serum markers of hepatic and renal injury in mice following 14 and 28- day exposure periods

Concentration (mg/kg)	ALT (U/I)		AST (U/I)		Urea (mg/dL)	
	14 days	28 days	14 days	28 days	14 days	28 days
NC	13.20 ± 0.67	19.32 ± 0.27	42.10 ± 1.88	51.00 ± 0.50	122 ± 12.4	180 ± 2.59
18.75	18.80 ± 0.22***	18.79 ± 0.73	47.00 ± 6.75*	55.40 ± 0.94	281 ± 0.00***	214 ± 10.80
37.50	15.60 ± 0.74*	18.57 ± 0.56	51.30 ± 0.90*	60.10 ± 1.80	257 ± 0.00***	249 ± 21.7
75.00	15.60 ± 0.46*	19.00 ± 1.05	51.80 ± 0.50*	56.00 ± 2.74	260 ± 4.66***	285 ± 50.7*
150.00	19.90 ± 0.00***	18.71 ± 0.76	50.00 ± 0.00	55.30 ± 0.75	265 ± 0.00***	246 ± 8.28
300.00	18.40 ± 0.63***	19.57 ± 0.57	47.80 ± 0.92	53.80 ± 0.75	351 ± 22.20***	312 ± 22.8**
PC	12.30 ± 0.65	18.46 ± 0.54	44.8 ± 0.75	55.60 ± 0.89	226 ± 18.6***	355 ± 2.74***

NC = Negative control (distilled water) PC = Positive control (cyclophosphamide - 20 mg/kg) *, **, *** Significant at p < 0.05, p < 0.01 and 0.001 respectively

Table 4.30 Effects of 1:1 mixture of AgNPs and CuONPs on serum markers of hepatic and renal injury in mice following 14 and 28- day exposure periods

Concentration (mg/kg)	ALT (U/I)		AST (U/I)		Urea (mg/dL)	
	14 days	28 days	14 days	28 days	14 days	28 days
NC	13.2 ± 0.67	19.32 ± 0.27	42.10 ± 1.88	51.00 ± 0.50	122 ± 12.4	180 ± 2.59
18.75	17.57 ± 0.14***	16.99 ± 0.57	47.80 ± 2.25	58.30 ± 3.75*	228 ± 34.7*	246 ± 26.9
37.50	17.14 ± 0.52 **	17.71 ± 1.24	51.50 ± 0.00**	56.50 ± 2.00	206 ± 0.00**	217 ± 23.5
75.00	17.50 ± 0.22**	17.85 ± 0.57	50.80 ± 0.00**	57.50 ± 1.50	311 ± 5.69**	278 ± 17.6 *
150.00	18.89 ± 0.59***	18.36 ± 1.70	49.50 ± 0.66**	56.00 ± 0.87	286 ± 6.21**	275 ± 8.28*
300.00	-	18.14 ± 1.05	-	54.10 ± 1.13	-	257 ± 9.33*
PC	12.3 ± 0.65	18.46 ± 0.54	44.8 ± 0.75	55.60 ± 0.89	226 ± 18.6***	355 ± 2.74***

NC = Negative control (distilled water) PC: cyclophosphamide (20 mg/kg) *, **, *** Significant at p < 0.05, p < 0.01 and 0.001 respectively

- Lost samples

CHAPTER FIVE

DISCUSSION

The application of nanoparticles is exponentially increasing in various areas of human endeavours and lifestyle but their possible impact on the environment and human health is yet to be fully understood. The applications of nanoparticles in drug delivery, biomedical devices, electronics, personal care products, wastewater treatments, food and food packaging, and agrochemicals have increased their potential release and raised toxicological concerns in the environment (Doak and Dusinska, 2017). The various stages of NPs lifecycle such as production, transportation, research and development, fabrication, consumer use and inappropriate disposal can lead to human exposure and its release into the aquatic and terrestrial environments (Scown *et al.*, 2010; Gupta *et al.*, 2017). Once the NPs are intentionally or inadvertently released into the environment they interact with biological, chemical and physical environmental entities and may lead to deleterious consequences in human, animal and plant health in both terrestrial and aquatic environments (Gupta *et al.*, 2017).

Currently, the manufacture and use of AgNPs and CuONPs are rapidly increasing mainly due to their antimicrobial properties (Alaraby *et al.*, 2016; Chen *et al.*, 2015). The likelihood of these two NPs being combined together in consumer product and also their coexistence in the environment is an aspect of silver and copper(II) oxide nanoparticles' toxicity that is scarce in literature. Co-exposure of silver and copper(II) oxide nanoparticles in biological systems could lead to antagonistic, synergistic or additive genotoxicity. Hence, the need for the safety of AgNPs, CuONPs and their 1:1 mixture in organisms (in plant system, aquatic and terrestrial models) from different ecological habitats. In this study, evaluation of DNA damage and systemic toxicity of silver and copper(II) oxide nanoparticles, and their 1:1 mixture was carried out in *Allium cepa* (a representative plant system), *Clarias gariepinus* (an aquatic vertebrate) and *Mus musculus* (mammalian terrestrial representative).

5.1 Physicochemical characterisation of silver and copper(II) oxide nanoparticles and their 1:1 mixture

Detailed physicochemical characterization of nanomaterial prior to genotoxicity testing is a vital aspect of nanomaterial safety assessment (Warheit, 2008; Doak *et al.*, 2012). This is necessary because of the varying distinctive properties of NPs such as size, surface area and chemistry, crystallinity and properties in different media which have effects on its biological and chemical reactivity and consequently its toxicity (Koedrith *et al.*, 2014). Also, of importance is the use of the correct or appropriate methods for analysis for determination of the NPs properties.

In this study, the TEM analysis was used for the characterisation of the dry NPs size and this showed that they were of sizes below 100 nm as stipulated by the manufacturer. However, different levels of agglomeration were observed in Milli Q water and tap water from the dynamic light scattering (DLS) analysis. For the AgNPs and the NPs 1:1 mixture, Z- average size (hydrodynamic size) was higher in dechlorinated tap water compared to distilled water. This increase in agglomeration of the NPs in solution may be due to the presence of ions in the dechlorinated tap water. This is in accordance with the report of Yue *et al.* (2015) that higher ionic strength and chloride concentrations increased agglomeration of AgNPs at different concentrations. Agglomeration state, bioavailability, dissolution rate and uptake of NPs are factors that affect toxicity. Similarly, the slight decrease in hydrodynamic diameter of CuONPs might be due to the differences in properties of the tap water such as pH and hardness which undermine hydrodynamic diameter (Sohn *et al.*, 2015). The observed zeta potentials for AgNPs, CuONPs and their 1:1 mixture shows that the NPs were unstable both in Milli Q water and dechlorinated tap water and could be responsible for varying toxicity responses. A NPs suspension is said to be unstable when zeta potential is between +30 and – 30mV (Keller *et al.*, 2010). Also, the high values of polydispersity index (PDI) show a moderate dispersion of particles sizes corroborating the large hydrodynamic size showing agglomerations of the NPs in the water media.

5.2 Cytogenotoxicity of silver and copper(II) oxide nanoparticles and their 1:1 mixture in root cells of *Allium cepa*

The role of the plant in both aquatic and terrestrial environment is very important. They serve as primary producers in the ecosystem. They convert sunlight energy to organic food substances. Thus, they are good representative for the evaluation of environmental fate and effects of chemical/substances in the terrestrial environment. Reports on the effect of NPs on plants shows that they may accumulate in crops and/or increase concentrations of the constituted metal of nanomaterials in the fruits or grains of a particular plant (Gardea-Torresdey *et al.* 2014; Gupta *et al.*, 2017). The accumulation of NPs can cause harmful effects on the yield and productivity of plants, alter the nutritional value of food crops, and also transfer them across the trophic levels of the food chain (Zhang *et al.* 2012). There is insufficient information on the genotoxicity of AgNPs and CuONPs and none on their 1:1 mixture. Moreover, the *Allium cepa* assay is a veritable assay that correlates well with other mammalian assay (Fiskejö, 1985). In this study, the potential cytogenotoxic effect of AgNPs, CuONPs and their 1:1 mixture was assessed in the root tips of *A. cepa*. The *A. cepa* assay provides for analysis of chromosomal aberrations and mutagenicity assessment in higher plants, as well as mitotic index and cytotoxicity (Tedesco and Laughing house, 2012).

Mitotic index (MI) as a cytological parameter gives information about the total number of dividing cells which is a good indicator of the cytotoxicity of the test agent and the chromosome aberration identifies aneuploidic and clastogenic effect of the test substances (Fiskesjo *et al.*, 1997; Leme and Marin- Morales, 2009).

The significant increase in mitotic index observed in the AgNPs – exposed root tips compared to the negative control at both exposure periods are indications of increases in cell division which shows that AgNPs is capable of stimulating cell division which can be harmful to the cell, thereby leading to disordered proliferation and consequently tumour/cancer development (Leme and Marin – Morale, 2009). However, the observed decrease during the recovery period compared to the genotoxicity group shows the tendency of the root tips to recover mildly from damage following withdrawal from exposure to NPs. The observed increase in MI is in concert with the

reports of Pesnya (2013) that AgNPs increased MI of *A. cepa* root tips at 50 mg/L. Contrastingly, Kumari *et al.* (2009) observed a decrease in MI at 25 – 100 ppm. The differences might be due to the difference in preparation of NPs and exposure periods. Kumari *et al.* (2009) exposed the root tips to AgNPs for 4 h which is below the cell cycle period. Its cell proliferating property could also be an indication of its beneficial therapeutic use in diseases conditions requiring bone marrow cell regeneration.

On the other hand, the decrease/ total inhibition in MI induced by the CuONPs compared to the control indicates that CuONPs is cytotoxic. This suggests that it slowed down/arrested the progression of cell cycle. and could be as a result of CuONPs interference in cell cycle; thus, leading to inhibitory and antiproliferative effects in the root tips cells. This might be as a result of inhibition of S phase (DNA synthesis) progressing to M phase or blocking of the G2 phase of the cell cycle, preventing the cell from entering mitosis. This reduction may be associated with the blocking of the formation of various metabolites necessary for the sequence of mitosis or inhibition of microtubule by the NPs (Smaka-Kincl *et al.*, 1996). The total mitotic inhibition observed at higher concentration of CuONPs in the *A. cepa* root tips corroborates with the reports of Deng *et al.* (2016) who observed similar distinct inhibitory effect of CuONPs on root tips between 24 – 72 h; and distinct inhibitory effect on root elongation at the onion root tips. Similarly, Lalau *et al.* (2015) reported toxic effects of CuONPs on duckweed plants with regards to growth inhibition, ultrastructure, and morphology as well as negative changes in photosynthetic pigments.

The NPs 1:1 mixture induced increases and decreases in MI at lower and higher concentrations respectively. This antagonistic interactive effect of silver and copper(II) oxide nanoparticles observed at 72h may be due to the selective absorption of Ag ions at lower concentration and Cu ions at a higher concentration which led to the arrest of cell cycle. Deng *et al.* (2016) reported that exposure to concentrations higher than 10 mg/L resulted in abnormally high accumulation of Cu ion in *A. cepa* roots compared to the lower concentrations.

Chromosome aberrations are changes in structure of chromosomes as a result of either breakage or exchange of chromosomal materials. In this study, chromosome

aberrations observed such as stickiness, spindle disturbances, nuclear abnormalities (micronucleated and binucleated cells) are indicators of genetic damage which have effect at either somatic or germline levels (Akinboro and Bakare, 2007). The most frequently observed aberration across the different groups of NPs and 1:1 mixture was sticky chromosomes indicating high toxicity which is irreversible and may lead to cell death (Fiskesjo, 1985). Other aberrations observed are related to spindle failures and these may have arisen due to inhibition of NPs on the microtubule tubulin polymerisation (Kuriyama and Sakai, 1974; Liman, 2013). The findings herein on CA in *A. cepa* root tips is in congruence with that of Kumari *et al.* (2009) and Pesnya (2013) who also reported CA in AgNPs exposed onions. Anaphase bridge could happen during the translocation of unequal chromatids thereby leading to chromosome mutation (El-Ghamery *et al.*, 2000; Luo *et al.*, 2004). C- metaphase suggest disturbance to microtubules which is reversible but could induce aneuploidy (Fiskesjo 1988; Odeigah *et al.*, 1997; Liman, 2013).

The DNA damaging activities of NPs in *A. cepa* could be associated with generation of free radicals from the surface of the NPs which is in contact with the meristematic cells. NPs possess large surface area to mass ratio and are very reactive when exposed to aqueous media. They can also adsorb free radicals to their surface in addition to releasing free radicals (Flower *et al.*, 2012). They may also induce cross linking between DNA and proteins as well as other damage. The increase in CA supports the clastogenicity of AgNPs, CuONPs and their 1:1 mixture in *A. cepa* root system. The observed genotoxicity can lead to genomic instability which is a predisposing factor and first step in carcinogenesis (Patlolla *et al.*, 2012).

5.3 Cytogenotoxicity and systemic toxicity induced by silver and copper(II) oxide nanoparticles and their 1:1 mixture in *Clarias gariepinus*

The result of MN induction in *C. gariepinus* shows that AgNPs, CuONPs and their 1:1 mixture significantly induced the formation of MN in peripheral erythrocytes of *C. gariepinus* following 14 and 28 - day exposure periods This suggests that the NPs and 1:1 mixture are clastogenic and/aneugenic. MN test which has MN formation as genetic endpoint (biomarker) is a widely applied test in the investigation of genetic

damage that occurs during the lifespan of the cell. MN is formed in the process of cell division as a result of a clastogenic (chromosome breakage) or aneugenic event (lagging chromosomes) during the metaphase stage of the cell division (Bolognesi and Cirillo, 2014). Furthermore, this suggests that the NPs were able to cross cellular membrane barriers probably through endocytosis to interact directly or indirectly with DNA during mitosis (Magdonelova *et al.*, 2014) in the kidney which is the main haematopoietic organ in fish.

Tested NPs might have gained access to bind chromosome or chromatin through nuclear pores or after the dissolution of the nuclear membrane (if large or aggregated particle size). Consequently, once inside the nucleus they can damage genetic material through interactions with DNA and histone proteins (Cavallo *et al.*, 2012). An indirect mechanism of damage could be via interaction of NPs with the mitotic spindle apparatus, centrioles or associated proteins (Magdonelova *et al.*, 2014). Huang *et al.* (2009) reported that long-term exposure to TiO₂ NPs disturbed mitosis by interfering with chromosome segregation, centrosome duplication and cytokinesis leading to abnormal multipolar spindle function, chromosome alignment and segregation needed for the process. Furthermore, the release of ions from NPs might have contributed to DNA damage. Ag⁺ and Cu²⁺ are transition metals that have been proved to catalyse intracellular ROS generation via Fenton reaction (Kruszewski *et al.*, 2011; Fu *et al.*, 2014). The increased MN frequency in peripheral blood of the exposed fish indicates potential genome instability which could lead to reduced fitness, genetic disorder and loss of aquatic diversity. The MN induction by AgNPs, CuONPs and their 1:1 mixture is in agreement with the recent reports of Sayed (2016) and Khan *et al.* (2017a) who demonstrated that AgNPs increased MN frequency in *C. gariepinus* and *Labeo rohita*. However, Sayed (2016) reported that AgNPs was lethal at 100 mg/L concentration. This observed difference could be as a result of differences in the preparation of NPs dispersions which is also an important factor that determines NPs toxicity. In this study NPs was suspended in distilled water while Sayed (2016) dispersed AgNPs in PBS which might have affected dissolution rate of the NPs and different ionic concentration and hence difference in toxicity. Also, the frequency of MN induction in CuONPs exposed fish is in agreement with the *in vitro* report of Karlsson *et al.* (2008) that CuONPs was more toxic compared to some selected NPs.

Interaction factor analysis of the NPs 1:1 mixture on MN induction showed an antagonistic interaction. This suggests that the AgNPs and CuONPs or their ions may have competed for binding sites in exerting their effect in the biological medium. Studies have shown that NPs release ions which contribute to cytotoxicity and genotoxicity of NPs (Beer *et al.*, 2012; Li *et al.*, 2017). Ag⁺ cation is monovalent and referred to as a chemically soft species like mercury and lead because it binds strongly to proteins and nucleic acid and forms insoluble complexes with RNA and DNA. Cu²⁺ on the other hand is divalent and higher in the electrochemical series. Ghandour *et al.* (1988) reported that Ag⁺ has a higher affinity for cells than Cu²⁺ hence; these could lead to competitive binding. This might be a probable reason for the observed antagonistic interactive genotoxicity.

Haematological assessment of fish blood is a vital tool in diagnosing health status of fish in relation to xenobiotics exposure. Alterations in haematological parameters in fish may be an adaptive response of the fish to physiological and immunological changes to stress and hypoxia following exposure to the NPs (Ayoola *et al.* 2014). In this study, PCV, RBC, Hb and platelet counts decreased in fish exposed to the NPs compared to the negative control. A decrease in RBC parameters and platelets suggests a reduction in oxygen supply in the fish circulatory system, anaemia and thrombopenia. This could be a resultant effect of inhibition of erythropoiesis or destruction of peripheral blood cells (Malhotra and Srivastava, 1979). Consequently, all these might have led to the observed alterations in MCV, MCH and MCHC which are indices signifying size of red blood cells, the amount of haemoglobin per cell and weight of haemoglobin in relation to blood volume respectively (Cheesbrough, 2005). The white blood cells are known to play important role in immunological defence mechanism in fish. The decreases in WBC and a concomitant increase in lymphocyte counts suggest potential immunomodulation in the NPs -exposed fish. Decreases in WBC could be due to the suppressive or inhibitory effect of the NPs on haemopoiesis in the fish. The increase in lymphocytes indicates stimulation of the immune system in response to the NPs. The observed haematological alterations in fish exposed to AgNPs, CuONPs and their 1:1 mixture in this study are similar to those reported by Alkaladi *et al.* (2015) and Rajkumar *et al.* (2016) following exposure of *Oreochromis niloticus* and *Labeo rohita* to zinc oxide and silver

nanoparticles respectively. Observed haematological alterations indicate stress and impairment in fish physiological functions as a result of exposure to the NPs.

Histopathological assessment provides information about organism's health. It provides important information about sub-lethal stress induced by xenobiotics in target tissues and mechanism of injuries (Wu and Zhou 2013). The observed histopathological alterations in the NP exposed fish suggest compromised cellular structure and function of the skin, gill, and liver. The skin of aquatic vertebrates acts as the first line of physical barrier against foreign bodies and maintenance of internal milieu (Groff, 2001; Esteban, 2012). The observed hyperplasia of metabolically active epidermal skin cells (keratinocytes, goblet cells, and alarm cells) largely suggests stress to fish and this might be an adaptation NPs exposure (Lee *et al.*, 2012). The increase in number or size of the specialized epidermal cells might lead to alteration in the chemical composition of the cells. This report is in accordance with those of Ostaszewska *et al.* (2016) on Ag and Cu nanoparticles, and Federici *et al.* (2007) on TiO₂NPs in Serbian sturgeon and rainbow trout respectively who also observed hyperplasia in gills of the exposed fish.

The gills serve the important function of osmoregulation and respiration in fish. Also, mucous sections on the fish gills serve as the first line of defense in the gill epithelium to xenobiotics (Shephard, 1994). Analysis of gill morphology showed primary lamella hyperplasia, degeneration of secondary lamella. This is an indication of damage to the gill as it has direct contact with fish environment hence, they are prone to damage. This further suggests that NPs were able to bypass the mucus secretion due to their small size. Lamella degeneration suggests impairment of gill function which could lead to the inefficient supply of oxygen and systemic hypoxia (Shaw *et al.*, 2012). Al-baruity *et al.* (2013) and Wu and Zhou (2013) also reported similar findings in gills of rainbow trout and Japanese medaka exposed to CuNPs and AgNPs respectively. The liver is an important organ for metabolism and detoxification of xenobiotics. Damage to the liver will lead to malfunctioning of the fish in its response to toxic waste products. Vacuolation observed in the hepatocytes of the NPs –exposed fish has been reportedly found proximal to neoplasm in brackish water catfish from a polluted water source (Olarinmoye *et al.*, 2011). This is an indication of tumour formation as tumour prevalence has been associated with increased vacuolation of hepatocytes

(Augspurger, 1994). These observed lesions might be due to the accumulation of the silver and copper ions or nanoparticles in the hepatocytes. These metals are capable of inducing the formation of reactive oxygen species which might consequently lead to damage of cellular macromolecules and structures.

The induction of antioxidant enzymes and molecules represents cellular defense mechanism against cellular toxicity. Alterations in levels of the oxidative stress biomarkers further support the observed histopathological lesions in skin, gills, and liver of the fish. The significant elevation of MDA (lipid peroxidation) in the liver of fish exposed to AgNPs and CuONPs indicates oxidative damage. This suggests the formation of reactive oxyradicals which are capable of damaging DNA, protein, and lipids (Pandey *et al.*, 2003; Magdonelova *et al.*, 2014). The decrease in level of MDA induced by the nanoparticle 1:1 mixture is an indication of lower level of lipid peroxidation and could be as a result of the activities of the antioxidant enzyme in the prevention of cellular injury by ROS (Ubani- Rex *et al.*, 2017). The observed increases in GSH levels in exposed fish compared to the control may be a response to reactive oxygen species (ROS) by NPs/metal ions. AgNPs, for instance, has been reported to increase ROS in the cell (Asharani *et al.*, 2009).

The SOD-CAT antioxidant system is known to be the first line of defense against oxidative stress. The SOD catalyses scavenging of superoxide radicals to water and hydrogen peroxides while CAT detoxifies the hydrogen peroxide to harmless compounds. However, in this study, AgNPs and CuONPs increased levels of SOD while CAT decreased. This might be due to synthesis of new SOD (Khan *et al.*, 2017b), and an adaptive response to the high production of superoxide anion leading to synthesis of new SOD while CAT reduction might be due to depletion of CAT in response to ROS production. Decrease in level of CAT in response to superoxide anions formation has been reported to inhibit CAT activity in fish exposed to polluted water (Pandey *et al.*, 2003). Corroborating the alterations in the levels of antioxidants enzymes/molecules with levels of MDA therefore suggests that AgNPs, CuONPs and their 1:1 mixture induced ROS subsequently leading to oxidative stress insult in the liver tissues.

This study is probably the first, showing the cytogenotoxic potentials of silver and copper(II) oxide nanoparticles as well as their 1:1 mixture using the piscine micronucleus assay, haematological and histopathological analysis of target organs of *C. gariepinus*.

5.4 Cytogenetic and systemic toxicity induced in *Mus musculus*

The acute toxicity test did not show mortality in the mice exposed to both AgNPs and CuONPs at oral dose of 600 mg/kg bw which indicates that the mice were able to clear or neutralize the NPs from the system and therefore shows that the LD₅₀ is greater than 600 mgkg⁻¹. This is in concert with the report of Maneewatanapinyo *et al.* (2011) where authors did not observe mortality at 5 000 mg/kg oral dose for colloidal silver (10 – 20 nm) after 14 days in both male and female mice.

The use of body and organ weight measurement has been reported as a sensitive indicator of drug/chemical toxicity. In the absence of morphological changes, organ weight comparison between the treated and control animal groups gives useful information about the toxicity of the chemical to target organ (Piao *et al.*, 2013)

In this study, the observed decrease in the % body weight at 300 mg/kg bw AgNPs and 75 mg/kg bw CuONPs compared to the control indicates toxic effect of the NPs. This might be due to reduced palatability of diet resulting from modulation of hormones responsible for food consumption (Bailey *et al.*, 2004). Several studies showed non-significant changes in body weight of AgNPs exposed mice (Park *et al.*, 2010; Xue *et al.*, 2012; Adeyemi and Faniyan, 2014). However, Shahare and Yashpal, (2013) reported significant decreases in mice orally administered AgNPs (3 – 20 nm) at 14 and 21 days of exposure. The authors reported that the damage observed to microvilli might have led to the decrease in nutrient absorption. Also, differences in preparation of NPs might be a contributing factor and suspension media. The observed increase in spleen weight at 150 mg/kg AgNPs bw at the 14th day could be due to stimulated increase in T and B cells (Weaver *et al.*, 2017)

The *in vivo* mammalian micronucleus (MN) test is an important genotoxicity test among a battery of assays required for genotoxic risk assessment of chemicals and physical agents (Schmid, 1975; Krishna and Hayashi, 2000). It detects cytogenetic

damage on chromosome or mitotic apparatus of erythroblasts. Its endpoint of assessment of damage is the formation of micronucleus (OECD, 2013). It is an appropriate test assessing potential adverse effects of exposure on terrestrial environment.

This study showed that 14 - day oral administration of AgNPs, CuONPs and their 1:1 mixture induced a significant increase in the frequencies of micronucleated polychromatic erythrocytes (MNPCE) in the bone marrow of mice. This indicates that the NPs and their 1:1 mixture have clastogenicity and or aneugenic potential and uncontrolled or continuous exposure could lead to genomic instability and consequently deleterious diseases, malformations such as cancer (Shugart, 2000). The NPs and their ions might have had direct or indirect interactions with the genetic materials or spindle fibre thereby leading to the observed genotoxic effect (Beer *et al.*, 2012; Magdolenova *et al.*, 2014). Studies have shown that metal and metal oxide nanoparticles are capable of eliciting toxicity via the Trojan Horse – type mechanism (Karlsson *et al.*, 2008; Park *et al.*, 2010b; Di Bucchianico *et al.*, 2013; De Matteis *et al.*, 2015).

Cell membranes are good barriers for toxic ions. However, these NPs gain access into the cell via endocytosis through the lysosomes and may facilitate the transportation of metal ion into the cell through Trojan horse type mechanism whereby the trapped NPs in vesicles such as lysosomes release ions which damage cell membrane (Park *et al.*, 2010a; Foldbjerg and Autrup, 2013). The large agglomerates of both NPs and their 1:1 mixtures (which are greater than 100 nm) observed in this study might have released metal ion which elicited the observed DNA damage.

AgNPs, CuONPs and their 1:1 mixture induced increased frequencies of MNPCEs at the two higher concentrations in the 28 -day experiment. This is in line with observations of Song *et al.*(2012) who reported increased frequencies of MN peripheral blood erythrocytes in mice administered single doses of AgNPs and CuONPs (3mg/mice). It is also in tandem with the reports of Dobrzyńska *et al.*(2014) and Patlolla, *et al.* (2015b) who also observed DNA damage in form of MNPCEs up to a 4th week (28 days) after a single intravenous injection of AgNPs and 5 days IP injection of AgNPs. However, Kim *et al.* (2008; 2011) did not observe a significant

increase in MNPCEs in the bone marrow of treated mice after 28 days oral exposure and 90 days inhalation in the rats.

There are limited reports on *in vivo* genotoxicity of AgNPs and CuONPs. Also, the available results on genotoxicity are conflicting. The majority of the observed differences in the nanotoxicity results are due to the differences in particle size, preparation method, doses, and suspension medium. More importantly, the agglomeration tendencies of NPs which make dispersion difficult are confounding factors (Schluesener and Schluesener, 2013).

Nanoparticles have been reported to accumulate in vital organs of mammalian rodents such as lungs, brain, liver kidney, spleen testes after exposure either orally or intraperitoneally (Park *et al.*, 2010; Lee *et al.*, 2013; Privalova *et al.*, 2014). The frequencies of MNPCEs were observed to be significantly higher in the 28 days experiment compared to the negative control group. This is an indication of the NPs biopersistence in the mice body system. Dobrzyńska *et al.*(2014) also reported biopersistence and increased MNPCEs in mice 28 days post exposure to AgNPs. Similarly, Lee *et al.* (2013) observed the persistence of AgNPs up to 90 days, post oral exposure. The non – significant difference in the MN induction between the 14- and 28-days exposure experiments in AgNPs exposed mice suggest higher retention of AgNPs in the body system compared to CuONPs and the 1:1 mixture. The significant reduction in MNPCEs in both CuONPs and 1:1 mixture group might be an indication of clearance of the NPs from the system to a considerable extent or possible incomplete repair of genetic damage (Dobrzyńska *et al.*, 2014)

The ratio of PCE to NCE give an indication of cytotoxicity (Krishna, and Hayashi, 2000) in *in vivo* MN assays. The higher frequencies of PCEs observed in the AgNPs exposed mice shows it was not cytotoxic at both exposure periods. This is in accord with reports of Kim *et al.* (2009; 2011) and Dobrzyńska *et al.* (2014). CuONPs, on the other hand, was observed to be cytotoxic during the recovery period.

To the best of my knowledge this is the first report on the interactive genotoxic effect of both NPs 1:1 mixture in the mammalian bone marrow. The antagonistic genotoxic and cytotoxic interactive effect of the NPs 1:1 mixture observed corroborates the previously obtained results in other models at the lower trophic levels and other

ecological habitats (*Allium cepa* and *C. gariepinus*). This indicates that co-exposure to both NPs can result in reduction in genotoxic effect and in the activities/ levels of GSH, SOD and MDA.

The increased white blood cells observed in mice following exposure to the AgNPs is possibly as a result of immunogenic response or disturbance in signalling pathways and maturation of cells which affects red blood cells as well as division and development of other cells. This is in concert with the report of Sarhan and Hussein (2014) that AgNPs (20 – 60 nm) interacted with blood and its components leading to immunogenic responses, inflammation and changes in haematological parameters such as increased white blood cell and platelets after acute intravenous exposure in rat. Huang *et al.* (2016) similarly reported that AgNPs is capable of promoting lymphocyte proliferation although it also cytotoxic at low concentration.

Ag ion is capable of causing mitochondria perturbation through its interaction with thiol group of mitochondrial inner membrane and disruption of its function. NPs could also gain access into the nucleus following intracellular uptake, intracellular transportation to the nucleus and subsequent DNA damage. Ag ions could also release ROS from its surface interaction with membrane molecules. The AgNPs cytotoxicity may have occurred via down regulation of Erk pathway as observed in human leukemic cell line Jurkat (Huang *et al.*, 2016).

On the other hand, CuONPs and NPs 1:1 mixture was cytotoxic by inducing decrease in WBC with a concomitant immunogenic response of lymphocytes at 14 and 28 days. CuONPs cytotoxicity might have occurred through its ability to interact with mitochondrial membrane and thus inducing apoptosis. This is in congruence with other studies in A549, HeLa and MEF cells (Karlsson *et al.*, 2009; Wang *et al.*, 2012b).

Histopathological assessment of tissues/ target organ in nanotoxicity evaluation gives information about the localized effects of NPs living systems (Yang *et al.*, 2017). This information includes gross microscopic and ultra-structural changes as well as functional damage. The histopathological lesions observed in liver, kidney, spleen, and testis are all indicative that the NPs and their 1:1 mixture were able to cross the cell membranes and barriers such as blood testis barrier and penetrate the systemic blood

circulation thereby having adverse effect on cells of different organs and system in the exposed mice. This indicates cytotoxicity of AgNPs, CuONPs and their 1:1 mixture to the respective organs.

The findings of this study is in congruence with that of Park *et al.* (2010) and Dziendzikowska *et al.* (2012) who observed that release of AgNPs from the blood stream following oral ingestion of AgNPs led to accumulation and toxic effects such as cytoplasmic vacuolation, chronic inflammatory cells and dilated blood vessels in the liver kidney, hearts and lungs. Lee *et al.* (2016) also reported similar observation in liver kidney and spleen of rats. Other reports on CuNPs also showed histopathological changes of organs like liver, kidney and spleen following a single or short-term exposure (Chen *et al.*, 2006; An *et al.*, 2012; Manna *et al.*, 2012). The observed histopathological change corroborates the observed weight increases liver, kidney and spleen as well as the mild reduction in the relative testis weight – hyperplasia, vacuolation necrosis e. t.c. are indications of increase in cell mass.

The liver is the first target for absorbed materials from the gastro intestinal tract and is an important organ of biodistribution and also capable of accumulating large doses of NPs. The observed liver toxicity may be due to accumulation of the NPs and 1:1 mixtures. This could lead to inhibition of mitochondria respiratory chain that produces energy for the cell or generation of ROS as a result of inflammatory oxidative, genotoxic, cytotoxic event and induction of apoptosis (Lee *et al.*, 2013; El Mahdy *et al.*, 2015). Also, the observed histopathological lesions might be due to activation of macrophages (neutrophils, kupffer cells) directly or indirectly by the NPs which eventually results in release of inflammatory mediators, growth factors and ROS. According to Patlolla *et al.* (2015a) AgNPs accumulation in liver exhibited mild infiltration of inflammatory cell in the periportal area.

Similarly, the observed lesions in the kidney buttress the role of the kidney as the excretory organ for clearance of NPs. The NPs might have passed through the liver and be excreted into the bile thus inducing tubular damage observed in the kidney tubule. This is in consistence with Liao and Liu (2012) who reported the presence of necrotic cells in proximal renal tubules following exposure to 50 – 200 mg/kg doses of CuNPs.

The spleen is an important organ of immunologic response. The observed hepatosplenomegaly indicates the toxicity of the NPs and their 1:1 mixture to both spleen and liver simultaneously which is in concert with the existing reports on NPs toxicity. Damage observed within testis as an organ of reproduction is also indicative of potential reproductive toxicity of both NPs and 1:1 mixture to effective transfer of life from parents to offspring. This could lead to a decrease in viability and low fertility or compromise in developing foetus.

In this study, levels of oxidative stress parameters suggest that the main mechanism of AgNPs genotoxicity is via oxidative stress. The AgNPs significantly increased the concentration of MDA (lipid peroxidation). This is an indication of oxidative damage in the mice liver. Different studies have suggested that AgNPs produces ROS which leads to cellular damage such as DNA damage and apoptotic cell death (Choi *et al.*, 2010; Tiwari *et al.*, 2011; Kim and Ryu, 2013; Li *et al.*, 2017)

Furthermore, the decrease in GSH, SOD and CAT induced by AgNPs further suggests oxidative stress which may be due to inhibition of GSH synthesizing enzymes or increased demands for GSH in conjugation with electrophilic molecules or depletion of the antioxidant enzymes (Rogers *et al.*, 2008; Kim and Ryu, 2013). This is in concert with the reports of Patlolla *et al.* (2015a; 2015b) which identified that AgNPs generated ROS *in vivo* and eventually led to oxidative stress as previously highlighted in cytogenotoxicity of and systemic toxicity in fish. This is of utmost concern for NPs induced toxicity.

On the other hand, the decreased MDA observed in the CuONPs treated mice shows that it did not successfully induce ROS as the antioxidant defense enzymes/molecules were concomitantly altered. SOD which is the first line of defense for scavenging ROS was depleted and the levels of GSH and CAT decreased. However, the oxidative damage increased though not statistically significant at the 28th day. ROS generation might not be the main mechanism for oxidative damage in the CuONPs group. The NPs and the Cu²⁺ might be responsible for observed genetic damage via direct interaction with chromosome during cell division. Magdolenova *et al.* (2014) explained that NPs could induce genetic damage via direct interaction with genetic material or indirect primary and secondary genotoxicity through ROS and

consequently oxidative stress. In this study, CuONPs formed large agglomerates (DLS results – above 2000 nm). CuONPs may have gained entrance into the cell through endocytosis and end up releasing Cu^{2+} and also made contact with the genetic material when nuclear membrane dissolves during mitosis. This is described as coordination effect. Furthermore, the presence of NPs in the periphery of the nucleus may block cellular transcription and translation machinery (Singh *et al.*, 2009). In addition, the Cu^{2+} may lead to cytoplasmic mRNA degradation by interacting with mRNA stabilizing proteins which contain metal responsive domains or with molecules cellular signaling molecules thereby inducing DNA damage and cell death (Miller *et al.*, 2010; Soenen *et al.*, 2010). The ROS generation within the recovery period could be due to the release of Cu^{2+} from accumulated CuONPs in the mice tissue (Chang *et al.*, 2012).

This is probably the first time the interactive effect of the 1:1 mixture on the oxidative stress parameters is being reported in mice for the first time. The pattern of oxidative stress seems to be similar to the pattern observed in the silver nanoparticle group. The increased levels of MDA concomitantly induced an increase in the antioxidant defense (SOD and CAT). However, with increased ROS, SOD was depleted and CAT increased to balance the oxidative damage. The increased ROS production might be responsible

Serum hepatotoxicity and renal toxicity biomarkers were also assessed using AST and ALT and Urea concentrations. Serum ALT and AST are indicators of cellular insults to the liver while the serum urea concentration gives information of kidney health status. The increased levels of ALT and AST across the different groups (both NPs and 1:1 mixture) at 14 days are indication of the acute hepatotoxic effect of both NPs and their 1:1 mixture. Other reports have also shown that NPs oral exposures have toxic effect on the liver function (Kim *et al.*, 2008; Tiwari *et al.*, 2011; Lee *et al.*, 2013; Patlolla *et al.*, 2015a; Lee *et al.*, 2016; Canli and Canli, 2017).

The ALT activity which is a more specific enzyme for liver status decreased at 28th day compared to the control. This indicates that there is a compromise in the liver metabolic function. This could be as a result of cellular damage involved in aging. This corroborates reports that low ALT level is associated with aging and frailty and might mediate mortality (Le Couteur *et al.*, 2010; Ramaty *et al.*, 2014).

The significant increase observed in the serum urea level in the NPs treated mice suggests potential acute renal toxicity of AgNPs, CuONPs, and their 1:1 mixture. The kidney is an important organ for excretion of waste product. A compromise in kidney function could lead to deleterious complications and mortality. The results of the genotoxicity, oxidative stress biomarkers, and histopathology, as well as the liver and kidney function tests, corroborate one another. They all point towards the potential genotoxicity of AgNPs, CuONPs, and their 1:1 mixture via either generation of ROS or from direct NPs or their metal ions or direct interaction with genetic materials and important proteins involved in cell division

The findings of this study show cytotoxic and genotoxic effects of sublethal concentrations of silver and copper(II) oxide nanoparticles and their 1:1 mixture at different ecological trophic levels in both aquatic and terrestrial environments. This is an indication that release of the NPs into the environment at different stages in the life cycle of the NPs can lead to detrimental genetic defects in different life forms (plant and animals) in the ecosystem. The DNA damage observed may lead to mutation and consequently increased risk of cancer and other genetic anomalies. The antagonistic interactive genotoxicity of the NPs 1:1 mixture observed in the three bio - model shows that co - exposure to the NPs in both plants and animals as well as aquatic and terrestrial environments may lead to similar damaging effect of the genetic materials.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

The potential genetic and systemic damage by AgNPs, CuONPs and their 1:1 mixture, were investigated using bio models as representatives of three ecological trophic levels. The *Allium cepa* assay showed that AgNPs was not cytotoxic but genotoxic in *A. cepa* root tips. However, the root system recovered mildly in the absence/ withdrawal of AgNPs. On the other hand, CuONPs was highly cytotoxic and more genotoxic with mild recovery from cytotoxicity only. The mixture (1:1) of AgNPs and CuONPs was also cytotoxic and genotoxic and showed antagonistic and synergistic interactions respectively at both exposure and the recovery period

The cytogenotoxicity assessment of both NPs and their 1:1 mixture using *C. gariepinus* showed the cytogenotoxic potential of AgNPs and CuONPs at higher concentrations following long and short-term exposure which was mediated via oxidative stress and direct interaction of NPs/ ions with the DNA. Also, the DNA damage overwhelmed the DNA repair mechanism at both exposure periods. Similarly, the 1:1 mixture of NPs had an antagonistic genotoxicity.

Oral exposure to both NPs and their 1:1 mixture in mice had genotoxic effects. Also, oral exposure to both NPs and control had potential renal and hepatotoxic effect via

oxidative stress and possibly through accumulation metals ions and NPs. The toxicity of AgNPs and Copper(II) oxide nanoparticles depends on type and preparation methods of NPs as well as exposure routes and targets organ/system.

Contributions to knowledge

The following are the contributions to knowledge.

1. *Allium cepa* when exposed to Silver (Ag) and Copper(II) oxide(CuO) nanoparticles showed proliferative and mitodepressive effects respectively while their 1:1 mixture showed both proliferative and mitodepressive effects.
2. Exposure of *A. cepa* root tips to Ag, CuO and their 1:1 mixture showed cytogenetic damage when exposed with insignificant recovery.
3. *Clarias gariepinus* and *Mus musculus* when exposed to Ag, CuO nanoparticles and their 1:1: mixture at sub lethal concentration showed genetic and systemic disruptions in their somatic tissues and organs via generation of oxidative stress
4. The mixture (1:1) of AgNPs and CuONPs had antagonistic interactive effect on cytogenotoxicity in the somatic cells of *A. cepa*, *C. gariepinus* and *M. musculus*

Recommendations

There is a need for further assessment of genotoxicity at individual cell levels (in target organs) for more specific determination of mechanism of damage. Also, a paradigm shift to toxicogenomic approach will enable a faster, more elaborate and accurate information for investigative and predictive toxicology.

Molecular profiling of DNA sequence of models of tropical origin like *Clarias gariepinus* will enhance the identification and determination of molecular biomarkers (expressed transcripts and proteins) related to molecular toxicity. In addition, Nigerian Governmental agencies in charge of health and environmental protection should be properly funded and proactive in the testing of new chemical and materials such as nanoparticles in the country.

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APPENDIX

I. Ethical approval for the study

UNIVERSITY OF IBADAN, IBADAN, NIGERIA
DEPARTMENT OF VETERINARY PATHOLOGY



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19th January, 2015.

Dr. A.A. Bakare,
Dept. of Zoology,
University of Ibadan,
Ibadan.

RE: APPLICATION FOR ETHICAL APPROVAL ON UI-ACUREC/014/1

On behalf of the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC), I write to grant you an **Ethical Approval** for a study on the "*In Vivo Evaluation of Genotoxicity and Mutagenicity of Metal and Metal Oxide Nanoparticles in Animal Models*" **strictly as outlined in your final proposal** submitted for assessment.

Please quote UI-ACUREC/App/2015/005 as reference for this approval.

You are to note that UI-ACUREC reserves the right to monitor and conduct compliance visit to your research site without previous notification.

Thank you.

Prof. V.O. Taiwo
Chairperson, U.I ACUREC

Cc: Dean, FVM
Director, Research Management Office

II. Mortality record of fish exposed acutely to AgNPs and CuONPs during the acute toxicity test

Time	No of animals per group	Control		CuO NP				Ag NP			
				100mg/L		500mg/L		10mg/L		100mg/L	
		Replicate 1	Replicate2	Replicate 1	Replicate2	Replicate 1	Replicate2	Replicate 1	Replicate2	Replicate 1	Replicate2
24h	10	0	0	0	0	0	0	0	0	1	0
48h	10	0	0	0	0	0	0	0	0	0	0
72h	10	0	0	0	0	1	1	2	0	0	0
96h	10	0	0	2	1	0	0	1	0	1	0