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TOXICOLOGIC EVALUATION OF SILVER NANOPARTICLES

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This is to certify that this study is fully adequate in scope and quality as a thesis for the degree of doctor of philosophy.

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APPROVAL:

In the opinion of the above Examining Committee Members appointed by the Executive Council of the Institute of Health Sciences, this thesis was found to satisfy all the requirements as a thesis for the degree of Doctor of philosophy and therefore is accepted at the meeting of the Institute Executive Council of Health Sciences.

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ÖZET

Charehsaz, M. Nanopartilüllerin Toksikolojik Değerlendirmesi. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmasötik Toksikoloji Programı Doktora Tezi, Ankara, 2014. Gümüş nanopartiküller benzersiz fizikokimyasal ve bakteri öldürücü özellikleri nedeniyle çeşitli ürünlerde kullanılmaktadır. Artan yoğunlukta kullanılan bu nanopartiküller halk sağlığını tehdit eden maruziyet risklerini de beraberinde getirmektedirler. Gümüş nanopartiküllerin bazı çalışmalarda canlı organizma üzerine istenmeyen etkileri olabileceği öngörülmektedir. Ancak reprodüktif ve gelişim toksisiteleri ile ilgili cevaplandırılması gereken birçok eksik nokta mevcuttur. Bu çalışma kapsamında gümüş nanopartiküllerin (0.2, 2 ve 20 mg/kg/gün) ve gümüş nitratın (20 mg/kg/gün) gebelik döneminde anne sıçanlar ve bunların yeni doğan yavruları üzerinde olası toksik etkileri incelendi. Bu amaçla bu nanopartiküller gebeliğin 7. ve 20. günleri arasında ağız yoluyla anne sıçanlara verildi. Doğumdan sonrası 2inci gün anneler ve yavrular dekapite edildi ve kan ve doku örneklerinin alınmasını takiben gümüş seviyesi bu örneklerde atomik absorbsiyon spektroskopisi yöntemi ile ölçüldü. Vücut ve organ ağırlıkları, ALT, AST ve IL-6 seviyeleri, oksidatif stres parametreleri, genotoksisite ve histopatolojik inceleme gümüş nanopartiküllerin güvenlik değerlendirilmesi için incelendi. Sonuç olarak; gümüş nanopartiküllerin 20 mg/kg/gün dozuna kadar fertilite ve reprodüktif parametreler yönünden olumsuz bir etkisi bulunamadı. İncelenen anne ve yavru dokularının gümüş içeriği kontrol grubuna göre daha fazla bulundu. Ayrıca sonuçlar nanopartikül ve iyonik gümüşün anne ve yavrularda oksidatif strese neden olabileceğini gösterdi, ancak sonuçlar iyonik formda anlamlıydı. Annelerin beyin dokusunun histopatolojik incelemesi nanopartikül ve iyonik gümüşün hipokampal skleroza neden olabileceğini gösterdi. Bu çalışma ışığında, daha önce bildirilen deney hayvanlarında gözlenebilen hiçbir yan etki göstermeyen en yüksek dozun (NOAEL) yeniden belirlenmesi gerektiği değerlendirildi.

Anahtar Kelimeler: Gümüş nanopartikül, reprodüktif, oksidatif stres, dağılım

ABSTRACT

Charehsaz, M. Toxicity Evaluation of Nanoparticles. Hacettepe University Health Sciences Institute PhD Thesis in Pharmaceutical Toxicology, Ankara, 2014. Silver nanoparticles (Ag-NPs) are widely applied in several products due to their unique physico-chemical properties as well as bactericidal effects. The extensive application of Ag-NPs has raised concerns about their potential toxicity. The evaluation of toxicity of Ag-NPs in some previous studies has revealed the adverse effects of these NPs on living organisms. However, very little is known about the potential toxicity of Ag-NPs on dams and offspring during the prenatal exposure. In the present study, reproductive/ developmental toxicity of orally administered Ag-NPs (0.2, 2 and 20 mg/kg/day) and $AgNO_3$ (20 mg/kg/day) was investigated in rats from gestation day 7 to 20. At 2 days after parturition, dams and pups were sacrificed and Ag level in maternal as well as pups organs was measured by Atomic absorption spectrometry. For safety evaluation of administered Ag-NPs, body and organ weights; ALT, AST and IL-6 levels; oxidative stress parameters; comet assay and histopathological examination were performed. As a result, no treatment related effects were found for the reproductive parameters including pregnancy length, maternal weight gain, and number of implants, fetal weight and litter size up to 20 mg/kg daily dose. Ag content of all investigated tissues was found higher in Ag-NPs and $AgNO_3$ treated groups than control in both dams and pups, indicating the possible transfer of Ag from pregnant rats to the fetus. The data suggest that Ag in nanoparticulate and ionic forms can affect oxidative stress in both dams and related offspring. However, this ability was just observed significantly when Ag was administrated to pregnant rats in ionic form. Histopathological examination of brain tissue revealed the high incidence of hippocampal sclerosis in both Ag-NPs and $AgNO_3$ treated dams. As a conclusion, the NOAEL values reported in previous studies may be needed to adjust in the light of findings from this study.

Keywords: silver nanoparticles, developmental, oxidative stress, distribution

CONTENTS

ABBREVIATIONS

AAS	Atomic absorption spectrometry
Ag	Silver
Ag-NPs	Silver nanoparticles
$AgNO_3$	Silver nitrate
ALT	Alanin aminotransferase
AMI	Acute Myocardial Infarction
AOT	Anionic surfactants
AST	Aspartate aminotransferase
BBB	Blood-brain barrier
Caltech	California Institute of Technology
CAT	Catalase
CTAB	Cetyltrimethylammonium bromide
DLS	Dynamic Light Scattering
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GD	Gestational day
G6PD	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced Glutathione
GSSG	Oxidized glutathione
HBV	Hepatitis B virus
HIV	human immune-deficiency virus
IC	Inhibitory concentration
IL-6	Interleukin-6
IBM	International Business Machines corporation’s
LOAEL	Lowest observable adverse effect level
MDA	Malondialdehyde

MDCK	Madin Darby Canine Kidney
MT	Metallothionein
NADH	Nicotinamide dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	nanogram
nm	nanometer
NNI	National Nanotechnology Initiative
NO	Nitric oxide
NOAEL	No observable adverse effect level
NPs	Nanoparticles
3-NT	3-nitrotyrosine
$^1O_2$	Singlet oxygen
OD	Optical density
OECD	Organization for Economic Cooperation and Developments
$OH^{\bullet}$	Hydrogen peroxide
8-OHdG	8-hydroxydeoxyguanosine
$ONOO^-$	Peroxynitrite
3-PDP	3-pentadecylphenol
PEG	Polyethylene glycol
PND	Postnatal day
P-4VP	Poly(4-vinylpyridine)
PVA	poly(vinyl alcohol)
PVP	Polyvinyl pyrrolidone
SDS	Sodium dodecyl sulfate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TNF	Tumor necrosis factor
$O_2^{-\bullet}$	Superoxide
SOD	Superoxide dismutase
STM	Scanning tunneling microscope
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet–visible

XRD	X-ray diffraction
YUDETAM	Yeditepe University Medical School Experimental Research Center

FIGURES

TABLES

1. SCOPE AND PROJECT AIM

Nanotechnology which is also known as a new technological revolution in the 21st century has been expanding rapidly in recent years. Nanoscience involves the study of materials on the nanoscale level between approximately 1 and 100 nm. Nanotechnology is the science of studying material in a tiny scale which could have a huge impact on our lives. Nanomaterials are currently used in different areas such as electronics, biomedicine, pharmaceuticals, cosmetics, environmental analysis, and catalysis as well as material sciences, due to their generally high reactivity and surface area and unique physical and chemical properties (1, 2).

Among the commercially available nanosized materials, nanosilver is one of the nanomaterials with the highest degree of commercialization, as roughly 30% of all products currently registered in nano-product databases claim to contain nanosilver (3). The remarkably strong antimicrobial activity is a major reason for the recent increase in the development of products that contain nanosilver (4, 5).

Beside the rapid proliferation and growing commercial use of engineered nanomaterials, these materials possess size-related physicochemical and biological properties differing significantly from larger matter. Therefore, it is necessary to be aware of these new materials safety and potential hazards (6, 7).

Some limited studies on the toxicity of silver nanoparticles (Ag-NPs) claim that these nanoparticles (NPs) cause oxidative stress, which then drives inflammatory, genotoxic and cytotoxic outcomes (6). However, compared to the vast application of Ag-NPs, there are very few studies investigating the toxic effects of these NPs, and even fewer containing data on special fields of toxicology such as reproductive/developmental toxicity, endocrine disruption and immunotoxicity.

Among the previous studies, some suffer from inadequate characterization of administrated Ag-NPs and/or lack of silver ion group for comparison,

leaving a crucial knowledge gap on the toxicity evaluation of Ag-NPs in comparison to silver ions. Furthermore the excessively high doses utilized within some studies undermine the relevancy of the findings.

In this present study the repeated-dose developmental toxicity of synthesized and well characterized citrate-capped Ag-NPs was investigated in Sprague Dawley rats orally. Additionally, to find out whether Ag-NPs toxicity emanates from their small size, or whether their release of silver ions or a combination of both, a group of animals were treated with silver nitrate ($AgNO_3$) to investigate the similarities or differences between soluble and NP silver.

To assess tissue distribution, total silver (Ag) contents were determined in a broad range of organs in moms and offspring.

Oxidative stress parameters were evaluated in blood, brain and liver of moms and offspring.

Hepatotoxicity was monitored by analysis of alanin aminotransferase (ALT) and aspartate aminotransferase (AST) levels in plasma.

Interleukin-6 (IL-6) as an inflammatory parameter and metallothionein (MT) as essential biomarker in metal-induced toxicity were also measured in plasma samples of dams and offspring.

The histopathological analyses were conducted in a broad range of organs in moms and pups.

To evaluate the genotoxicity of the Ag-NPs the alkaline Single-cell gel electrophoresis (Comet) assay technique was performed in lymphocytes of moms. Furthermore, the effect of Ag-NPs on progesterone, estradiol and oxytocin were investigated.

The outcomes of this project will help to increase the understanding of adverse responses to Ag-NPs exposure and the mechanisms behind observed responses. Also it is well known that fetuses have few protective

mechanisms, indicating that they could be more vulnerable to NPs than adults and thus, the findings of this study will serve to assess the possible adverse effects of Ag-NPs on developmental processes.

2. INTRODUCTION

2.1. Nanotechnology, Definition and Historical Development

Nanoscience or nanotechnology is the study of phenomena, properties and responses of matter on a molecular and atomic scale. This molecular level investigation is usually conducted at a range below 100 nm. The “nano” is a prefix denoting 10^{-9} which comes from *nanos*, a Greek word meaning dwarf. In the case of nanotechnology, it refers to things that are one-bilionth of a meter in size. To get a sense of the nano scale, a human hair is about 100,000 nanometers thick and lining only ten hydrogen atoms together will make up one nanometer (8, 9). According to National Science Foundation and National Nanotechnology Initiative, nanotechnology is the ability to understand, control and manipulate matter at the level of individual molecules and atoms, as well as at the supramolecular level involving clusters of molecules (in the range of about 0.1 to 100 nm), in order to create materials, devices and systems with fundamentally new properties and functions due to their small structure (10).

Nanotechnology is the science of studying material in a tiny scale which could have a huge impact on our lives. Besides the socioeconomic, environmental and survival needs, the intrinsic human need for intellectual development is a key driver for discoveries and advancements in science and technology. Today, nanotechnology is one of the leading scientific and technological fields with great potential since it combines knowledge from the fields of physics, chemistry, biology, medicine, pharmaceutical sciences, informatics and engineering.

Nanotechnology is an emerging and dynamic field with over 50,000 articles published annually in this field in recent years and more than 2,500 patents being filed at major patent offices such as the European Patent Office (11).

Among nanomaterials, NPs play an important role in nanotechnology advances. In general, NPs can be categorized into carbon-based materials

such as fullerenes and carbon nanotubes and inorganic NPs including the ones based on metal oxides (zinc oxide, iron oxide, titanium dioxide and cerium oxide etc), metals (gold, silver and iron) and quantum dots (cadmium sulfide and cadmium selenide).

A variety of products containing engineered NPs have been produced and some are in commercial use in very broad range including metals, ceramics, polymers, smart textiles, pharmaceutical products, cosmetics, biomedicine, electronics, paints, chemicals, food and environmental analysis (1).

Although nanotechnology is a modern concept and has ambitiously been called the next industrial revolution but it has been noted that the ancient artists also took advantage of using small scale particles in their works of art. The Lycurgus cup, for example, is an outstanding decorative treasure dated to the fourth century. This late Roman cut vessel is made of a glass that changes color when light is shone through it. The glass contains gold-silver alloyed NPs, which are distributed in such a way to make the glass look green in reflected light but, when light passes through the cup, it resembles a ruby red (Figure 2.1) (12).

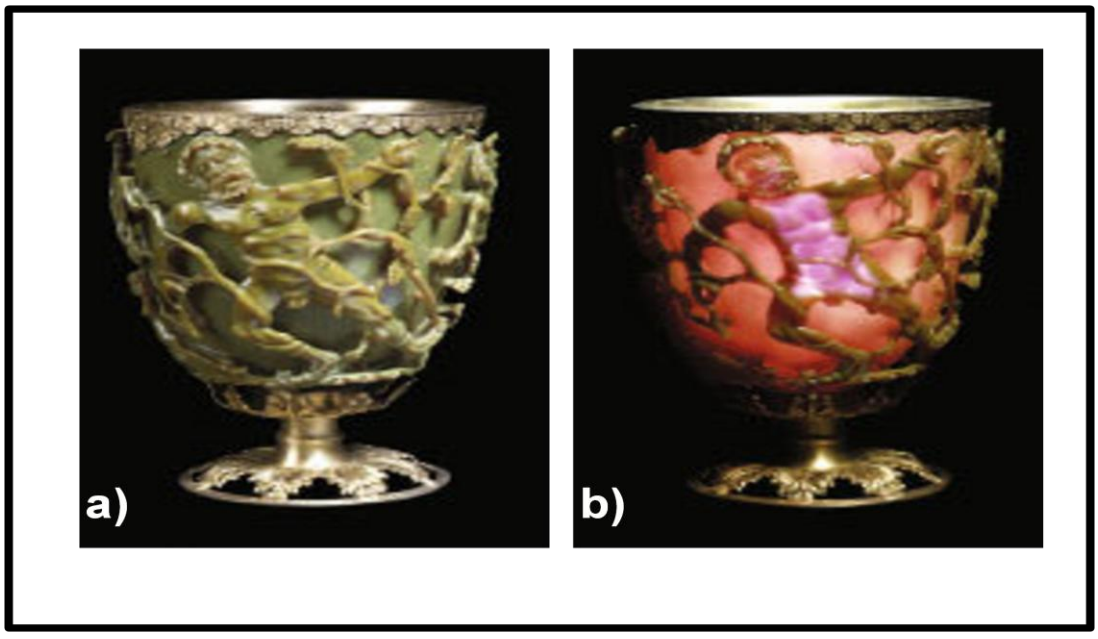


Figure 2.1. The Lycurgus cup in reflected (a) and transmitted (b) light (12).

Damascus steel swords, are another example of ancient artifacts which were created using nanomaterials. Damascus steel was a type of steel used in South Asian and Middle Eastern sword making industry between three and seventeenth century and is known for its impressive strength, shatter resistance and exceptionally sharp cutting edge. The steel blades contain nanowires and carbon nanotubes in their structures, which almost certainly enhances the material's properties (13).

The idea of nanotechnology as a modern concept was first time introduced, when Richard Feynman, a physicist at California Institute of Technology (Caltech), gave a talk called “There’s Plenty of Room at the Bottom” on 29th December 1959, at the annual meeting of the American Physical Society held at the California Institute of Technology.

"I am not afraid to consider the final question as to whether, ultimately-in the great future-we can arrange the atoms the way we want; the very atoms, all the way down! What would happen if we could arrange the atoms one by one the way we want them [….]"-Richard Feynman, 1959.

Feynman’s revolutionary vision made nanotechnology a buzzword, launched the global nanotechnology race and covered a wide range of concepts and opportunities, on which we work today in the field of nanoscience (14).

In 1974, a Japanese scientist, Norio Taniguchi of the Tokyo University of Science, used the term "nanotechnology" for the first time in his article “On the Basic Concept of Nanotechnology”.

In 1979, Eric Drexler encountered Feynman's provocative talk on atomic manipulation. From this moment, Drexler’s primary goal was to build upon the Feynman’s revolutionary foundation. He prepared his initial scientific paper on the subject, “Molecular Engineering: an approach to the development of general capabilities for molecular manipulation,” published in the prestigious scientific journal, Proceedings of the National Academy of Sciences, in 1981. Drexler’s publication essentially expanded the idea of

molecular manufacturing by integrating modern scientific ideas with Feynman's original concepts. Drexler published his first book "Engines of Creation: The Coming Era of Nanotechnology" in 1986. In this book, he presented that if atoms are viewed as small marbles, then molecules are a tight collection of these marbles that snap together depending on their chemical properties. When snapped together in the right way, these molecules could represent normal-scaled tools such as motors and gears. Drexler envisioned that these molecular machines would then be used as "assemblers" that could bring atoms together into a desired shape. Applying his vision of molecular manufacturing, Drexler claimed that coal can be turned into diamond and computer chips can be created from sand in theory. From these principles, he sensationally proclaimed in his book that nanotechnology, through the molecular manufacturing of assemblers, would revolutionize everything from biological science to space travel as shown in quote bellow from Engines of Creation book:

"Molecular assemblers will bring a revolution without parallel since the development of ribosomes, the primitive assemblers in the cell. The resulting nanotechnology can help life spread beyond earth- a step without parallel since life spread beyond the seas; it can let our minds renew and remake our bodies-a step without any parallel at all".

Drexler also coined the term "grey goo" which refers to an end of the world scenario in which self-replicating nanobots take over the world (15, 16).

Directly after the publication of his book, Drexler founded the Foresight institute. This institute was a research center to further study nanotechnology and its future. Drexler is no longer a member of this Institute.

Due to the publicity generated by Drexler's effort, scientists from all over the world began to have an interest in the field of nanotechnology. His vision of molecular manufacturing and assemblers has become, on one hand, a scientific goal and on the other, a controversial issue. For example, Richard Smalley, a professor of chemistry, physics and astronomy at Rice

university in Houston, criticized Drexler stating that chemistry is extremely complicated, reactions are hard to control and that assemblers were not physically possible and are science fiction (17). Nevertheless, Smalley said that he was a “fan of Eric” and that Engines of Creation influenced him to pursue nanotechnology (18). Because of the onset of academic criticism from scientists such as Smalley, nanotechnology evolved from Drexler’s vision to a broad systematical field.

In addition to the criticism of Drexler’s vision, nanotechnology got a boost in 1981 with the invention of the scanning tunneling microscope (STM), an instrument for imaging surfaces at the atomic level, by Gerd Binnig and Heinrich Rohrer at the International Business Machines corporation’s (IBM) Zurich research laboratory. In 1986, Binnig and Rohrer shared half of the Nobel Prize in physics for their design of the STM. This development led to the discovery of fullerenes in 1985 by Harry Kroto, Richard Smalley and Robert Curl, who together won the 1996 Nobel Prize in chemistry. Also the invention of STM led to the structural assignment of carbon nanotubes a few years later (19).

The first manipulation of atoms using STM was illustrated through Don Eigler’s revolutionary stunt at IBM in 1989. He used 35 xenon atoms on a nickel surface to spell out the IBM logo (20).

Since November 1996, several scientists from multiple agencies had been meeting informally to discuss nanotechnology. Then, between 1997 and 2000, a long term research vision and an international benchmarking of nanotechnology in academe, government and industry were developed and completed by more than 150 experts in United States (21).

On January 2000, President Clinton announced the U.S. National Nanotechnology Initiative (NNI). In his speech at Caltech, Clinton said, “My budget supports a major new NNI, worth half a million dollar. Caltech is no stranger to the idea of nanotechnology - the ability to manipulate matter at the atomic and molecular level. Over 40 years ago, Caltech's owner Richard

Feynman asked, "What would happen if we could arrange the atoms one by one the way we want them?" (22).

The NNI is a United States federal nanotechnology research and development program. NNI serves as the central point of communication, cooperation, and collaboration for all Federal agencies engaged in nanotechnology research, bringing together the expertise needed to advance this broad and complex field (10). The NNI has catalyzed global activities in nanotechnology and serves as a pattern for other programs. As a result of the NNI, the United States is recognized as the world leader in this area of nanotechnology. Nevertheless, NNI has been criticized for using ‘nano’ as a convenient tag to attract funding for a whole range of new science and technologies (10, 23). Certainly, one of the reasons of lightning development and global race in this field is the funding that nanoscience research has received from governments. Politicians’ interest and support from the field of nonoscience might have been due to the fact that they are willing to invest in something new and exciting especially those which have potential impacts on the energy and military sectors.

2.2. Fabrication at the Nanoscale

In general, as schematized in Figure 2.2, nanomaterials can be produced by either of the two techniques named, top-down or bottom-up. In top-down technology, material is produced in bulk and then shaped into a finished part through a variety of physical processes such as machining, casting, etching, rolling, forging and extruding. On the other hand, bottom-up technology which is often referred to as molecular nanotechnology, is manufacturing processes, in which the positions of individual atoms are controlled during fabrication by usually chemical processes (1, 24).

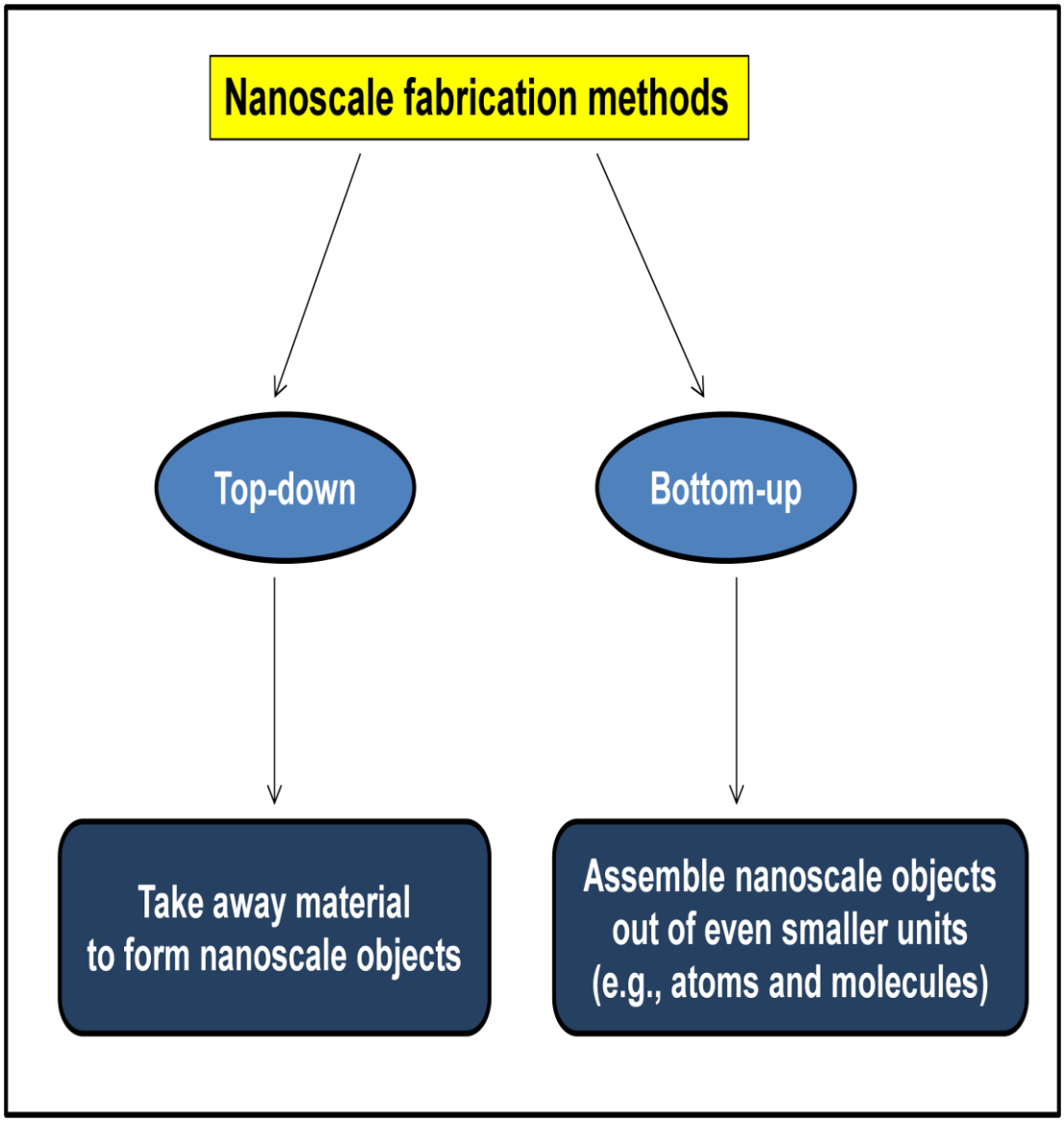


Figure 2.2. Top-down and bottom-up techniques of fabrication in nonoscale.

2.3. Nanotoxicology

Technology extends our abilities to change the world in order to suit us better. But the results of changing the world are often complicated and unpredictable. They can include unexpected benefits, unexpected costs, and unexpected risks. Therefore, anticipating the effects of technology is as important as advancing its capabilities.

Beside the rapid proliferation and growing commercial use of many different engineered nanomaterials, nanoscale dimension of these materials

possess physicochemical and biological nanostructure dependent properties that differs from the bulk properties of the constituent chemicals and compounds (6, 7). Therefore, it is necessary to be aware of these new materials safety and potential hazards. This requirement is resulting in an unavoidable growing interest in nanotoxicology, the study of the relationship between nanomaterials physicochemical properties and their toxic potential on living organisms and environment (8).

In 2005, a journal article titled ‘Nanotoxicology: An Emerging Discipline Evolving from Studies of Ultrafine Particles’ was published by Oberdörster et al. This influential article describes the demand for safety testing of nanostructured materials and also identifies nanotoxicology as an important field of research due to a number of biokinetic factors inherent to nanomaterials compared to bulk materials. In this journal article, it has been also demonstrated that nanoparticles (NPs) have the potential to be widely distributed in different organs through multiple routes of exposure (Figure 2.3) including inhalation, oral ingestion, dermal application and injection whether by intravenous or subcutaneous routes (25).

In 2005, the Nanomaterial Toxicity Screening Working Group was convened by The International Life Sciences Institute Research Foundation/Risk Science Institute. The objective of this group was to identify the key elements of a toxicity screening strategy for engineered nanomaterials (7).

The importance of this developing field became clear when, in 2007, a new scientific journal was published called Nanotoxicology, with its major focus being “how we can use the science of nanotoxicology to make nanomaterials safe in order to allow nanotechnology to reach its full potential in a safe and sustainable manner (26).

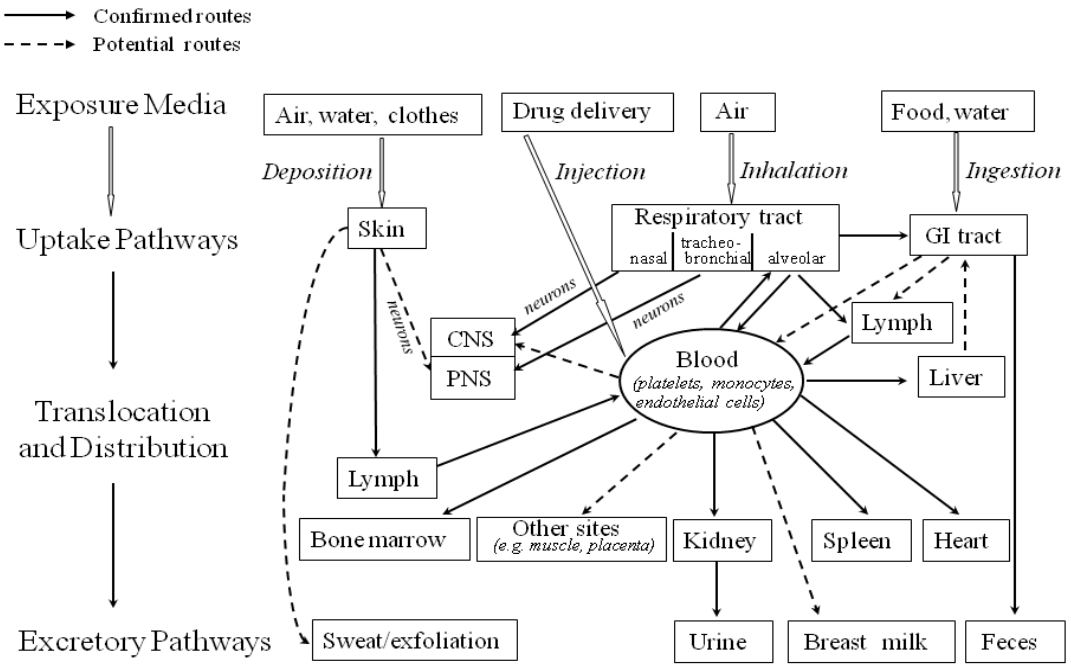


Figure 2.3. Exposure routes and distribution of NPs in humans (25).

In 2007 an Organization for Economic Cooperation and Developments (OECD), initiated the sponsorship program for the testing of manufactured nanomaterials. A major benefit of this program was the international collaboration and discussion that was facilitated around the crucial aspects of testing nanotoxicology to enable the safe use of manufactured nanomaterials (27).

2.3.1. Physicochemical Characterization in Nanotoxicology Study

The critical elements of a nanotoxicological screening strategy around NPs were developed by Oberdörster et al in a comprehensive article titled “Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy”. In this article, three key elements of the toxicity screening strategy including physicochemical characteristics, *in vitro* and *in vivo* assays were addressed (7).

Understanding the effect of physicochemical characteristics of NPs on their biological activities is a crucial part of nonotoxicology studies.

Characterization of test materials as supplied, as administered and during exposure represents the approaches to obtaining physicochemical information and provides useful baseline data on the material under test for all nanotoxicology investigations. Key properties to be characterized include: particle size and particle size distribution, agglomeration state, particle shape, crystal structure, chemical composition including impurities or contaminants, surface area; surface chemistry such as coatings or fictionalization, surface charge and porosity. In addition to the material characterization, data on potential human and environmental exposure, dose-response relationship, significance of dose, dose metric and biokinetics will be necessary to determine and evaluate the potential risks and safety hazards of nanomaterials following inhalation, oral or dermal routes of exposure (7, 8, 28).

2.4. Silver Nanoparticles

The metal NPs such as gold, silver, iron, zinc and metal oxides, which have many potential applications, have attracted great interest not only due to their large surface area to volume ratio, but also for their unique physico-chemical properties, including a high electrical and thermal conductivity, optical, magnetic and catalytic activity (4, 29, 30). Among the metal NPs, Ag-NPs have attracted more attention due to its antimicrobial and antifungal properties (4, 30-33).

Nanosilver is one of the largest and fastest growing classes of NPs in consumer products applications. Over 313 products utilizing nanosilver have entered the commercial market place around the world. According to a report on Ag nanotechnology commercial inventory, published in 2008, the health and fitness related products were found to be rank one of utilizing nanosilver followed by the other categories such as medical applications, electronics and computers. These widespread applications trigger scientific community to pay special attention to the research topic of Ag-NPs. Figure 2.4 shows a statistical data analysis of the trend in published research papers in this area within the last 14 years (4).

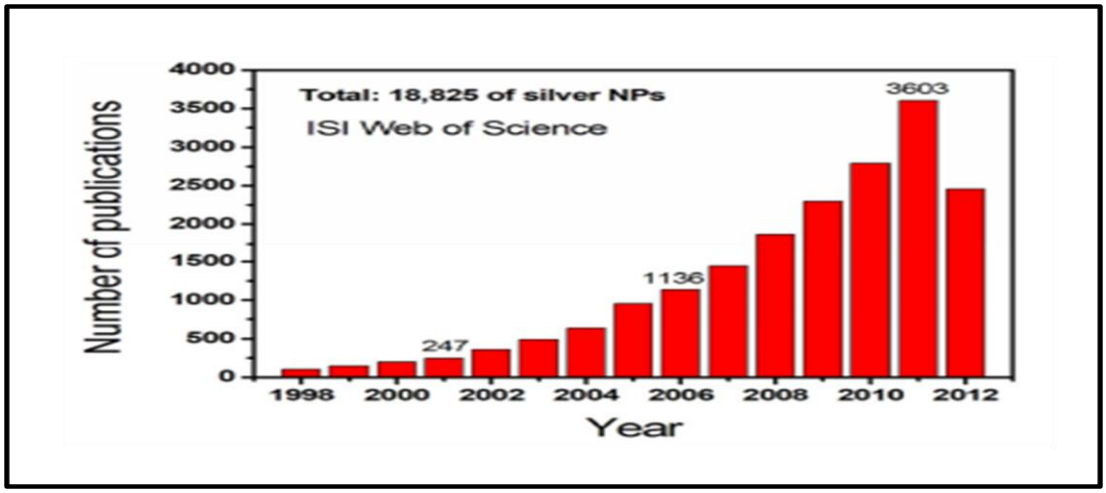


Figure 2.4. Trend in published research articles on the topic of Ag-NPs (4).

As shown in Figure 2.4, during the 10 years from 2001 to 2011, the number of published papers has grown by nearly 93% from 247 articles by 2001 to 3603 articles by 2011. Chemistry and materials science are the most popular topics of Ag-NPs research and China has the most published articles on this topic, followed by USA (4).

2.4.1. Antimicrobial and Antifungal Effects of Ag-NPs

Since the nineteenth century, Ag-based compounds have been used in many antimicrobial and disinfectant applications. It is well-known that Ag ions and Ag-based compounds are highly toxic to microorganisms. Ag is generally used in the nitrate (NO_3) form to induce antimicrobial effect, but when Ag-NPs are used, there is a remarkable increase in the surface area available for the microorganisms to be exposed comparing to $AgNO_3$ (33).

There are many researches on antibacterial (4, 30-46), antiviral (47-52) and antifungal (53-55) effects of Ag-NPs in various literatures.

Although the bactericidal effect of Ag-NPs against both aerobic and anaerobic microorganisms is very well known, the bactericidal mechanism is not clearly known and is a debatable topic. Some studies revealed that Ag-NPs attach to the bacterial cell membrane and disturb its proper functions such as permeability and respiration (33, 35). Also Ag-NPs are able to

penetrate inside the bacteria and cause further damage by possibly interacting with sulfur- and phosphorus- containing compounds such as vital enzymes and DNA which would lead to cell death (4, 33, 35, 44, 56). In one of the most cited articles, Kim et al demonstrated that the superior antibacterial properties of Ag-NPs are related to the formation of free radicals from the surface of them and subsequent free radical induced membrane damage (36). However, further researches are required on the bactericidal mechanism of Ag-NPs to thoroughly establish the claims.

The antiviral and antifungal activities of Ag-NPs have not yet been studied adequately. Few papers have been published that investigate the effects of Ag-NPs against viruses and fungus.

Although the antiviral mechanism of Ag-NPs has not been fully known yet, most publications have suggested that Ag-NPs could bind into outer proteins of viral particles, resulting in inhibition of binding and the replication of viral particles in cultured cells. For example, Elechiguerra et al have reported that Ag-NPs interact with the human immune-deficiency virus-1 (HIV-1) via preferential binding to sulfur-bearing residues of the gp120 glycoprotein knobs, resulting in the inhibition of the virus from binding to host cells (47). In another study, Lu et al investigated the effects of Ag-NPs on the hepatitis B virus (HBV). This study showed that Ag-NPs could inhibit production of HBV RNA and extracellular virions in vitro (48).

Xiang et al used Madin Darby Canine Kidney Cell Line (MDCK) to determine the effects of Ag-NPs against influenza A virus. It was observed that Ag-NPs have efficient inhibitory activity on influenza A virus and the results suggested that application of Ag-NPs may be a novel clinical strategy for the prevention of influenza virus infection during the early dissemination stage of the virus (51).

Ag-NPs have also been revealed as potential biocide against fungal strains. For example, Kim et al studied the antifungal activities of Ag-NPs against Various Plant Pathogenic Fungi. The results indicated that Ag-NPs

have considerable antifungal activity, probably through destruction of membrane integrity (53).

2.4.2. Application Fields of Nanosilver

Ag base nanomaterials are utilized in many fields and they have major applications due to their unique physicochemical properties and particularly their antimicrobial effects.

The high surface energy and reactivity of nanosilver resulted in the use of Ag based nonomaterial to catalyze many reactions in industrial processes such as degradation, reduction and oxidation (57-60).

The high electrical and thermal conductivity as well as enhanced optical properties of nanosilver result in various applications in electronic equipment such as optical devices, sensors, inks for printed circuit boards, high density recording and data storage devices (60-64).

Ag-NPs are used as strong antimicrobial agents in a various range of applications and consumer products including textiles, plastics, food packaging, food supplements, air sprays, detergent, soap, shampoo, toothpaste, air filters, coatings of refrigerators, vacuum cleaners, washing machines, cell phones, toys, antimicrobial paints, home appliances and water purification systems (60, 65-73).

In the food industry, Ag-NPs are widely used and there are several examples of food packaging material containing Ag-NPs, which are commercially available (3).

In a study, Echegoyen and Nerin investigated NPs release (with different stimulant solutions and times) from three commercial nanosilver plastic food containers. Ag migration was observed for all samples studied, with the total Ag migration values ranging between 1.66 and 31.46 ng/cm^2 (74). Authors indicated that in all cases the total Ag migration was far below the maximum migration limits stated by the European legislation. However, it should be noted that nanosilver is currently not assessed by European food

safety authority and thus there is a lack of a specific legislation for nanosilver that takes into account the differences in toxicity with respect to the bulk material (3, 74).

Most importantly nanosilver has many medical applications including diagnosis, treatment, medical devices and pharmaceuticals.

Some reports indicate that, Ag-NPs based Surface-enhanced Raman spectroscopy can be used for biological imaging and detecting cancerous tissues with more sensitivity than common instruments (75, 76).

Nanosilver is widely used for coating medical tools as well as sterilization of materials in hospitals. It is also incorporated in medical textiles, wound dressings, medical masks, diabetic socks, medical catheters and contraceptive devices (60, 73). In orthopedics, nanosilver is used as additive in bone cement and prostheses (60).

One of the most therapeutic uses of Ag-NPs is treatment of wounds in the form of a topical water soluble gel formulation or wound dressing materials (77). In a study conducted by Tian et al, he investigated the wound-healing capabilities of Ag-NPs using a thermal injury mouse model. Results showed that, healing of burn wounds occurred more quickly when topically treated with Ag-NPs comparing to Ag sulfadiazine as a standard burn treatment (78).

Saliminasab et al reported that, Ag-NPs can enhance the sensitivity of the human cardiac troponin, a specific biomarker for diagnosis of Acute Myocardial Infarction (AMI), sensor which will lead to a highly specific sensing system in human blood samples for diagnosis of AMI (79).

Zhou & Wang reported that Ag-NPs can inhibit the proliferation of human lung cancer cells in dose and time dependent manner *in vitro* (80).

Soumya et al investigated the nanosilver based targeted drug delivery for treatment of cancerous cells. The results indicated that nanosilver carrier

has an enhanced permeability to enter into cancerous cells and does not touch the normal cells (81).

Liu et al constituted a flexible nanosilver tool kit for controlled Release of biologically active Ag which can be used in a variety of medical and consumer antimicrobial applications (82).

Some of other potential medical applications of nanosilver are: infusion ports, endovascular stents, urological stents, endoscopes, peritoneal dialysis devices, subcutaneous cuffs, dental materials and coating of contact lenses (73, 83).

2.4.3. Synthesis of Ag-NPs

There are an extensive number of synthesis methods of producing Ag-NPs that are available in various literatures. All these reported methods can be categorized as physical, chemical, and biological methods (4, 33).

Chemical Synthesis

The chemical methods, as a typically bottom-up technique, have been mostly used for production of Ag-NPs because of simplicity of these methods. The basis of this method is the reduction of Ag ions (Ag^+) in aqueous solutions of Ag compounds such as $AgNO_3$ with appropriate reducing agents such as sodium borohydride and sodium citrate (4, 33, 84). Generally, three main components including Ag^+ precursors, reducing agents and stabilizing/capping agents are involved in chemical synthesis process (4, 33). $AgNO_3$ is the most widely used Ag^+ precursor in this method due to its chemical stability and low cost.

Although by utilizing chemical techniques, homogenous and stable nanosilver suspensions can be produced, this method also has some disadvantages such as low feasibility for the massive production for industrial use and accumulation of residual chemicals in the NPs suspension at the end of the synthesis processes (60, 85). Some chemically synthesis methods of Ag-NPs in different literatures is summarized in Table 2.1.

Table 2.1. Summary of some chemically synthesis methods of Ag-NPs.

Precursor Compound	Reducing Agent	Stabilizing/Capping Agents	Source
$AgNO_3$	Ascorbic acid	Daxad 19	Sondi et al, 2003 (84)
		SDS*	Chaudhari et al, 2007 (86)
		Starch	Iyer et al, 2007 (87)
		PEG*	
		P-4VP*	
	Sodium borohydrid	Polyelectrolytes	Radziuk et al, 2007 (88)
		PEG	
		Sodium borohydrid	Maneewattanapinyo et al, 2011, (89)
		Oleic acid	Seo et al, 2007 (90)
		PVP*	Van Dong et al, 2012 (91)
		PVA*	He & Kunitake, 2006 (92)
		Hexadecyl amine	Kuila et al, 2007 (93)
	Trisodium citrate	Trisodium citrate	Lee & Meisel, 1982 (94)
	Trisodium citrate and Hydrazine	SDS and Trisodium citrate	Guzman et al, 2009 (95)
	Hydrazine	silicic acid	Kang et al, 2011 (96)
		SDS	Zhang et al, 2008 (97)
	Ethylene Glycol	PVP	Bregado-GutieRrez et al, 2008 (98)
			Wiley et al, 2007 (99)
	Formaldehyde	PVP	Hsu & Wu, 2007 (100)
	Aniline	CTAB*	Hussain et al, 2011 (101)
Silver sulfate (Ag_2SO_4)	Sodium borohydrid	AOT* and SDS	Mandal et al, 2005 (102)
	3-PDP	3-PDP*	Swami et al, 2004 (103)
Silver perchlorate ($AgClO_4$)	Ethylene Glycol	Ethylene Glycol	Jacob et al, 2007 (104)
	dihydroxy benzenes	dihydroxy benzenes	Jacob et al, 2008 (105)
Silve oxide (Ag_2O)	Hydrogen gas	-	Merga et al, 2008 (106)

* SDS: sodium dodecyl sulfate, PEG: polyethylene glycol, P-4VP: poly(4-vinylpyridine), PVP: polyvinyl pyrrolidone, PVA: poly(vinyl alcohol), CTAB: cetyltrimethylammonium bromide, AOT: anionic surfactants, 3-PDP: 3-pentadecylphenol.

Physical Synthesis

For a physical approach as a top-down technique, Ag-NPs can be manufactured by methods such as thermal decomposition (107), laser ablation (108), gamma irradiation (109-111), electron irradiation (112), microwave processing (113), ultrasonic irradiation (114), sonochemical (115, 116) and electrochemical synthesis (117).

The physical approach can result in production of large quantities of Ag-NPs samples in a single process which is less toxic to the environment than chemical synthesis. However, primary costs for investment of equipment should be considered (4).

Biological synthesis

The problem with most of the chemical and physical methods is that they are extremely expensive and also involve the use of toxic and hazardous chemicals which may pose potential environmental and biological risks. A quest for an environmentally sustainable and economically feasible synthesis process has led to biological approaches which are also known as green synthesis methods (4, 33).

Similarly to chemical methods, biosynthesis of Ag-NPs is a bottom-up approach by which the reducing and stabilizer components are replaced by environmentally friendly agents such as fungus (118-120), plants or plant extracts (121-124), microorganisms (125-127) and enzymes (128, 129).

2.4.4. Characterization of Manufactured Ag-NPs

As mentioned previously, behavior of size distribution, polydispersity, aggregation sate, shape, surface chemistry, surface area and surface charge are crucial for toxicity and safety assessment of manufactured Ag-NPs (7, 130).

Some frequently used techniques for characterization of Ag-NPs in different matrix are UV-Vis spectroscopy, dynamic light scattering,

transmission electron microscopy, scanning electron microscopy and Energy dispersive X-ray diffraction spectroscopy.

UV-Vis Spectroscopy

UV-Vis spectroscopy offers the possibility of characterizing Ag-NPs because these NPs have a distinct peak in the UV-Vis region which differs from those of bulk material. UV-Vis spectroscopy is a useful, relatively cheap and easy method which allows estimation of size, concentration and aggregation level of NPs (131).

Dynamic Light Scattering

Dynamic Light Scattering (DLS), also known as photon correlation spectroscopy, is one of the most popular methods providing information on the hydrodynamic diameter and aggregation levels of nanosilver in suspensions. DLS is capable of measuring particles in the size ranges from 1 nm to approximately 5 μm. Also most DLS instruments are capable to measure zeta potential which demonstrates the surface charge of NPs (132).

Transmission Electron Microscopy

Electron microscopy techniques, based on the application of an electron beam, have a much higher resolution than traditional optical microscopy, which makes them a popular option in visualization and characterization of NPs.

Transmission electron microscopy (TEM) images provide information on the size and shape of the NPs. TEM, with appropriate detectors and software, also gives a range of other data such as morphology, aggregation state, elemental composition, bonding and redox activity of NPs (131).

Energy-Dispersive X-Ray Diffraction Spectroscopy

X-ray diffraction (XRD) is an analytical technique used for the elemental analysis and characterization of samples. Usually, it is combined with TEM or scanning electron microscopy to give an element distribution of the Ag-NPs (131).

2.5. Toxicity of Ag-NPs

Considering the vast application of Ag-NPs in various products, it is necessary to understand and be aware of the potential risks and hazards associated with exposure to these NPs. This knowledge will be useful in managing risk in the future through the introduction of protective protocols or by substitution of these substances with alternative materials (6).

Some studies on the toxicity of Ag-NPs claim that the main toxicity mechanism of these NPs is their oxidative nature, which then drives inflammatory, genotoxic and cytotoxic outcomes (6).

2.5.1. Oxidative Stress and Antioxidant Defense Systems

Reactive oxygen species (ROS), reactive nitrogen species (RNS) and other diverse chemical species including superoxide ($O_2^{-\bullet}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), as well as nitric oxide (NO) and peroxynitrite ($ONOO^-$) are involved in a variety of biological phenomena such as pathogenesis of many diseases, mutation, carcinogenesis and aging.

These various radical species, either generated exogenously or produced endogenously, can adversely alter all biological macromolecules such as lipids, proteins and DNA (133-136). For example, membrane lipids are highly susceptible to ROS damage. A lot of oxygenated compounds particularly aldehydes such as malondialdehyde (MDA) are produced during the attack of free radicals to membrane lipoprotein and polyunsaturated fatty acids. Products of lipid peroxidation formed in the primary site can reach to

the other organs and tissues via the blood stream and provoke further lipid peroxidation resulting in cellular and tissue damage (133, 137, 138).

Also ROS can attack DNA at different positions and cause several types of damage such as modification of DNA bases, single and double DNA breaks, loss of purines, damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system (136, 138).

Fortunately, there are several defense mechanisms for protection against these reactive molecules under physiological steady state conditions. These defense mechanisms, as shown in Figure 2.5, include physical defenses, prevention mechanisms, repair mechanisms and antioxidant defense system. Among these various defense mechanisms, the antioxidant system is extremely important due to its direct removal of pro-oxidants and maximum protection for biological sites. This system contains antioxidant enzymes and low molecular weight antioxidants (136).

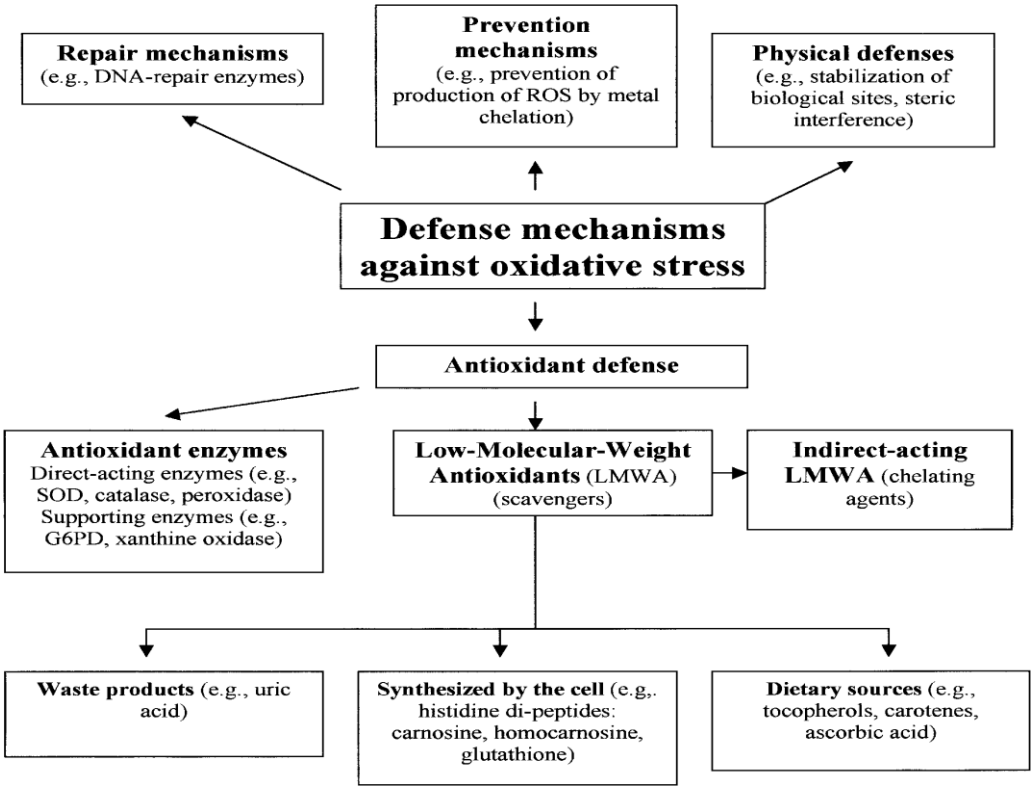


Figure 2.5. Classification of antioxidant defense mechanisms (136).

The antioxidant enzyme group is composed of direct acting proteins such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (133, 136).

SOD is an important scavenger enzyme which is present in all aerobic organisms and in all subcellular compartments susceptible to oxidative stress as well as extracellular fluids. There are several types of this enzyme which differ in their structure and metal cofactors.

Generally, the main role of SOD is catalyzing the dismutation of superoxide ($O_2^{\bullet -}$) into oxygen and hydrogen peroxide as shown by the following reaction.

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow{\textbf{SOD}} H_2O_2 + O_2$$

The resulting H_2O_2 as an end product of this dismutation reaction can be decomposed to water and oxygen by CAT and GPx (Figure 2.6).

Oxidation of CAT with H_2O_2 results in production of intermediate compound known as compound 1. A second molecule of H_2O_2 serves as an electron donor to compound 1 and results in the destruction of the two H_2O_2 molecules.

GPx is a key enzyme in the protection against oxidative stress and it can remove H_2O_2 even when it is present in low concentrations. As shown in the reaction below, GPx catalyses the reduction of H_2O_2 at the expense of glutathione (GSH) to water.

$$2GSH + H_2O_2 \xrightarrow{\textbf{GPx}} \text{glutathione disulfide (GSSG)} + 2H_2O$$

Also some other enzymes such as glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) function as secondary antioxidant enzymes. For example glucose-6-phosphate dehydrogenase supplies nicotinamide adenine dinucleotide phosphate (NADPH), which

provides the necessary reducing equivalent nicotinamide dinucleotide (NADH) for the regeneration of oxidized glutathione (GSSG) to the reduced form (GSH) (133, 136).

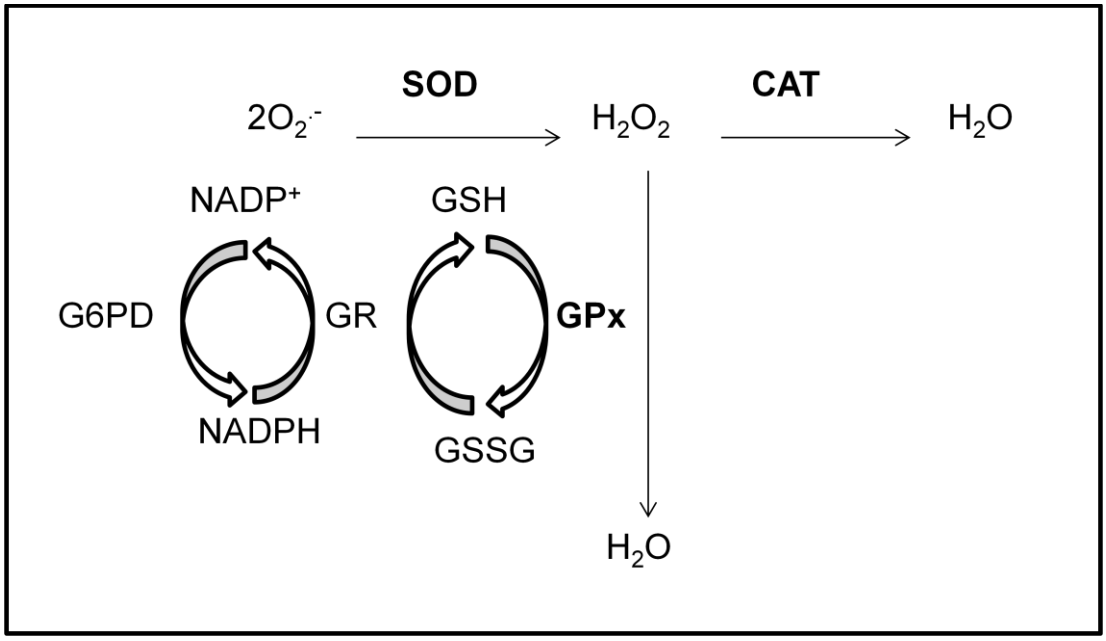


Figure 2.6. ROS scavenging function of antioxidant enzyme system (133).

Biomarkers of Oxidative Stress

The effects of oxidative stress, as well as information regarding the nature of the ROS and RNS, may be gleaned from the analysis of biomarkers of oxidative stress isolated from tissues and biological fluids.

The biomarker has been defined as a characteristic that is objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (139).

Generally, oxidative stress biomarkers can be divided in three categories. The first one consists of direct quantification of ROS and RNS such as free radicals. Although some techniques such as electron spin resonance allows the detection of relatively stable radicals, usually direct

detection methods are not yet applicable in clinical examination because of the reactive species' short half-life in biological samples and the need for expensive equipments.

The second approach is quantification of specific end products resulting from the interaction of the ROS with biological macromolecules which is the proof of ROS's prior existence. These molecular products remain in intracellular or extracellular compartments for a long time and generally considered more stable than ROS/RNS themselves. For example, peroxidation of membrane lipids by ROS is a very damaging phenomenon as it leads to alterations in the biological properties of the membrane. In the last stage of the peroxidation process, peroxides are decomposed to aldehydes like MDA which can then be measured in biological samples as an indirect index of oxidative stress (136, 140).

Another example is nitric oxide radical (NO) which is produced by the oxidation of one of the terminal guanido nitrogen atoms of L-arginine. The short half-life of NO makes it impossible to be accurately and directly measured. However, the final products of NO metabolism, nitrite and nitrate, can be measured in biological samples. The NOx is the sum of nitrite and nitrate concentrations served as an indicator of NO production (136, 141).

The reaction of NO with $O_2^{\bullet}$ results in production of peroxynitrite ($ONOO^-$), which can cause tyrosine nitration in protein and create 3-nitrotyrosine (3-NT), a valuable stable biomarker in tissues and fluids (136).

8-hydroxydeoxyguanosine (8-OHdG) is also another well know biomarker which represents cellular DNA damage caused by ROS generation (136, 140).

The third category of oxidative stress biomarkers consists of antioxidant enzymes that are associated with ROS scavenging such as SOD, CAT and GPx. In most cases, these enzymes are destroyed or modified and exhibit decreased or increased quantities after exposure to ROS (140).

2.5.2. Inflammation and Inflammatory Mediators

Inflammation is a local protective mechanism aiming to remove invading agents or initiate a healing process of damaged tissue. This mechanism must be regulated precisely, because deficiencies or excesses of the inflammatory response cause morbidity and shorten lifespan (142). Inflammation is mediated by a variety of inflammatory mediators, including a group of secreted polypeptides known as cytokines.

There are presently 18 cytokines with the name interleukin (IL). Other cytokines have retained their original biological description, such as tumor necrosis factor (TNF) (143). Cytokines involved in the inflammatory response can be divided into three subgroups: (i) pro-inflammatory cytokines, that initiate and enhance the cascade of events such as TNF-α, IL-1β and IL-8; (ii) IL-6 type cytokines, that induce the systemic actions; (iii) anti-inflammatory cytokines including IL-4, IL-10 and IL-13, that down-regulate the inflammatory response.

Among the IL-6 type cytokines, IL-6 is a prominent actor. IL-6 involves in the regulation of immune responses, the acute-phase response and hematopoiesis. IL-6 is released from activated monocytes in coordination with IL-1 and TNF-α thereby, one can induce production of another. Furthermore, IL-6 mediates communication between a large number of cell types by playing a role in the proliferation and differentiation of B lymphocytes and T lymphocytes and thus, IL-6 is considered a major immune and inflammatory mediator (144).

2.5.3. Review of Some Studies on Ag-NPs Toxicity

In Vitro Studies

In vitro models have been utilized in some studies to screen potentially toxicity effects of Ag-NPs in order to predict these effects on humans.

In general in vitro systems allow scientists to investigate specific cells or tissues without the distractions of having to look at a whole organism, which results in reducing the animals of animals examined in toxicology studies. Also *in vitro* approaches allow the clarification of the mechanisms behind toxicity at the cellular level. The primary endpoint of interest for *in vitro* toxicology is cytotoxicity, the ability of a compound to cause cell death by disrupting essential cellular processes such as metabolic rates, cell growth, gene transcription controlling functions and apoptosis. This endpoint is usually achieved by measuring cellular metabolism or membrane integrity. Such cytotoxicity tests are useful to define the intrinsic ability of a compound to cause cell death and the concentration range for planning further and more detailed studies. However, there are disadvantages with in vitro approaches, such as lack of reflecting real life, dosimetry mismatch and over simplicity (7, 145, 146).

In a study conducted by Hussain et al, the cellular response to Ag-NPs exposure was evaluated in rat liver epithelial cell line. It was identified that Ag-NPs induced a significant increase in cellular reactive oxygen species and decrease in GSH levels. However, it was not known whether it binds directly to GSH, or inhibits enzymes involved in GSH synthesis. In addition, a dose-dependent decrease in mitochondrial membrane potential and cell viability were also associated with particle exposure. It was not clear whether the toxicity was derived from Ag^{+} or NPs (147).

In another study, Cha et al investigated and compared the effects of Ag-NPs and silver microparticles on the human hepatoma cell line. The

results showed no significant cytotoxic effect and GSH content at any of the concentrations investigated (up to 2.4 µg/ml), when exposed for 72 hours to Ag-NPs or macroparticles. However, the DNA contents of cells decreased by 15% in the nanoparticle treated cells and by 10% in the microparticle treated cells, which they concluded to be indicative of more potent induction of apoptosis by the nanoparticles (148). Johnston et al criticized this study in their comprehensive article by which indicating that administrated concentrations are relatively low to accumulate in liver. They also stated that measuring GSH at one point of time is not appropriate and DNA content of cells is not an accurate analysis of apoptosis (6). In addition to Johnston et al's statement, it should be taken into account that cancer derived cell lines may behave differently to primary cells, so primary cells derived from the same organ should also be tested where possible.

Piao et al investigated the possible molecular mechanisms underlying the cytotoxic effects of Ag-NPs in human liver cells. Results indicated that Ag-NPs induced ROS generation and inhibited GSH resulted in cellular components damage, DNA breaks, lipid peroxidation and protein carbonylation. The GSH depletion mechanism of Ag-NPs, as suggested in this study, is inhibition of GSH synthesizing enzymes including GSH synthetase and catalytically active subunit of gamma glutamate cystein ligase. Also results of this study showed that Ag-NPs induced a mitochondria dependent apoptotic pathway resulting in the disruption of mitochondrial membrane potential. This mitochondrial disruption was followed by cytochrome c released from the mitochondria resulting in the activation of caspases 9 and 3 (149).

In another in vitro study, Sinko et al studied the effects of Ag-NPs on two important esterase enzymes acetylcholinesterase and butyrylcholinesterase. In this investigation, three different PVA stabilized Ag-NPs where prepared by the chemical reduction using trisodium citrate, hydroxylamine hydrochloride and borohydride. Results showed that all the three types of Ag-NPs were reversible inhibitors of acetylcholinesterase. Ag-

NPs prepared by hydroxylamine hydrochloride reduction were found to be the most potent inhibitor of both enzymes (150).

Arora et al studied the effects of Ag-NPs on primary mouse fibroblasts and liver cells. The Ag-NPs were in the size range of 7-20 nm and cells were exposed for 24 hours. Toxicity was evaluated by studying cellular morphology and mitochondrial function as an indicator of cytotoxicity as well as monitoring the effects on SOD, GSH and lipid peroxidation. Also localization studies were carried out by TEM after exposing the cells to Ag-NPs to find out whether these NPs are interacting with cells physically from outside or travelling inside the cells. Results indicated that cell morphology of primary fibroblasts and liver cells was not changed up to a concentration of 25 μg/mL and 100 μg/mL, respectively. The half maximal mitochondrial function inhibitory concentration (IC50) of Ag-NPs was found to be 61 μg/mL and 449 μg/mL for fibroblast and liver cells, respectively. In this study TEM observations indicated the presence of Ag-NPs in the mitochondria and cytoplasm of the cells exposed to a dose of half the IC50. The same dose of Ag-NPs appeared to result in enhancement of GSH and depletion of lipid peroxidation in fibroblasts as well as increase in SOD and GSH in liver cells. As suggested in this study, low levels of oxidative stress are considered to induce the expression of protective mechanisms. Higher concentrations were not investigated in this study. Arora et al concluded that, although Ag-NPs enter the primary cells and cause oxidative stress, the cellular antioxidant mechanisms prevent oxidative damage. Thereby, use of topical antimicrobial gel containing Ag-NPs for the treatment of burns and wounds is safe since primary cells possess much higher Ag-NPs tolerance than the concentrations encountered in this gel formulation (~20 μg/g) (77).

A recent study, conducted by Ucciferri et al, the toxicity effects of six types of NPs on human umbilical vein endothelial cells were investigated. In this study, the toxicity of NPs was assessed using a flow system to endothelial cells for better simulation of physiological conditions. As mentioned in this article, the importance of endothelial cells is barrier function

of these cells between the blood circulation and the surrounding tissues. Also the endothelium involve in a number of pathways such as lipid metabolism, vascular inflammation and modulation of vascular tone which are correlated with the onset of cardiovascular disease. Results of this study indicated that Ag-NPs were the most toxic among the investigated NPs. Ag-NPs trigged inflammation, activating an IL-8 dependent pathway as well as pro-apoptotic signaling leading to cell death. Also endothelial cells were shown to be more susceptible to Ag-NPs under flow conditions. Authors suggested that there is a requirement to develop physiologically relevant *in vitro* models for better understanding of NP hazard (151).

Some other in vitro studies also have indicated the release of several cytokines to be induced by Ag-NPs exposure to macrophages and human peripheral blood mononuclear cells (152-154).

AshaRani et al reported increased ROS generation resulting from the intercellular production of $O_2^{-\bullet}$ and H_2O_2 as well as mitochondrial damage associated with a depletion of ATP in starch-coated Ag-NPs treated human lung fibroblast and human glioblastoma cells. TEM imaging was used to show translocation of Ag-NPs into cellular compartments and Ag-NPs were found to distribute throughout the cytoplasm, inside lysosomes and nucleus. Also DNA damage was identified in this study by alkaline single-cell gel electrophoresis assay (155).

Single-cell gel electrophoresis (Comet) assay is a method for evaluating DNA lesions in individual cells. For the first time Ostling and Johansson developed this method by embedding cells in agarose on microscope slides followed by lysing and subjecting them to electrophoresis under neutral conditions enabling the detection of DNA breaks (156). A later modification, using alkaline conditions, was also developed by Singh et al (157). Under both neutral and alkaline conditions, increased DNA damage is visualized as an increased migration of genetic material (comet tail) from the nucleus (comet head) towards the anode (156, 157).

Comet assay results in the study carried out by AshaRani et al showed a concentration dependent increase in tail momentum comparing to control cells, which implies induction of DNA damage and chromosomal aberrations by Ag-NPs (155).

Chi et al also examined the genotoxicity of Ag-NPs using an acellular biochemical system. They concluded that Ag-NPs are only weak genotoxics themselves, as only Ag clusters can penetrate into DNA. But combination of Ag-NPs and detergent pollutants such as cetylpyridine bromide can enhance the genotoxic effect of Ag-NPs (158). However, it should be considered that such acellular systems only relate to direct genotoxicity and do not allow identification of the consequences of cell derived ROS or inflammation on gene integrity (6).

In another study, Ahamed et al investigated the genotoxicity of uncoated or polysaccharide-coated Ag-NPs in mouse embryonic stem cells and mouse embryonic fibroblasts. Both types of Ag-NPs induced DNA damage and apoptosis responses resulted in cell death in two types of mammalian cells. Also according to their results, coated NPs were more distributed throughout cell while agglomeration of the uncoated NPs restricted their cellular distribution. Consequently, coated Ag-NPs exhibited more severe damage than uncoated Ag-NPs (159).

AshaRani et al, reported that Ag-NPs cellular uptake occurs mainly through endocytosis by clathrin mediated process and macropinocytosis. Also authors revealed that the survival of cells from NP mediated damage depends on their ability to expel the NPs. Results of this study showed that cells are able to remove NPs through exocytosis process. However, this process occurs in a time dependent manner in cancer cells, which suggests chances of a prolonged Ag-NPs mediated stress and new hopes for preventing cancer cell metastasis. Furthermore, the up regulation of cryoprotective stress response genes like hemeoxygenase-1 and MT-1 showed that the cellular defense mechanisms are still active in normal cells (160).

Some other studies also revealed that exposure of cells to Ag-NPs, significantly express isoforms of MT related genes (161). MTs are considered to be essential biomarkers in metal-induced toxicity as involve in heavy metal homeostasis and detoxification (162, 163).

In Vivo Studies

The majority of reported nanotoxicology researches have focused on in vitro cell culture systems. However, the data from these studies could be misleading and may not represent the whole of organism responses.

Animal studies (*in vivo* models) are useful to understand the relationship between the physical and chemical properties of the nanomaterials and their behavior in living organisms, including humans. Such studies would provide basic information about the exposure routes through which NPs will enter the body and their kinetic parameters, as well as long-term chronic exposures which are practically impossible to achieve using in vitro systems (164).

Ag-NPs suspension is usually applied in animal studies and Ag-NPs in suspension have been described to release Ag^{+} (165, 166). However, it is still uncertain that whether Ag-NPs toxicity emanates from their small size, or whether their release of silver ions is responsible for any observed toxicity, or a combination of both (6).

In a study conducted by Kim et al, oral toxicity and tissue distribution of Ag-NPs (60 nm) were investigated, at 30, 300 and 1000 mg/kg/day concentrations, for a total of 28 days in rats. The results of micronucleus assay in this study suggest that Ag-NPs do not induce genotoxicity in rat bone marrow in vivo. However, the total Ag content of some organs such as liver, brain, kidney, lung and testes increased in a dose dependent manner, indicating that Ag-NPs were distributed in the tissues. Also a gender related difference in the accumulation of Ag was observed in the kidneys, with a twofold increase in the female kidneys comparing to males. The hematological examinations revealed that the dose dependent increase in

blood coagulation time enables the orally absorbed Ag-NPs to enter the blood circulation. Furthermore, significant dose dependent changes were found in the alkaline phosphatase and cholesterol in the concentrations above 300 mg/kg, which may be indicative of the liver damage. The histopathological examination revealed increased incidences of bile-duct hyperplasia, dilatation of the central vein and foci in liver (167).

In another article written by the same author, subchronic (90 days) oral toxicity of Ag-NPs (56 nm) was studied in rats. The dose levels were 30, 125 and 500 mg/kg/day in this study. There were no significant changes in food and water consumption during this study. However, significant decrease in the body weight of male rats was observed after 4 weeks of exposure. The results of histopathology, hematology, clinical chemistry and Ag distribution were similar to previous 28 day study. Authors concluded that liver is the target organ for Ag-NPs and also suggested that no observable adverse effect level (NOAEL) and lowest observable adverse effect level (LOAEL) of Ag-NPs are 30 and 125 mg/kg, respectively (168).

According to Johnston et al, the exaggeratedly high doses used within these two studies undermine the relevancy of the findings and thus consideration of human situation and reality of the experimental design is necessary to make the experiment more appropriate and relevant (6).

Loeschner et al. studied the distribution of Ag after 28 days repeated oral administration of Ag-NPs (14±4 nm) and Ag acetate to rats. As stated by authors, Ag acetate was chosen for comparison because it is a soluble salt containing a biocompatible counter ion. The daily dose of Ag in the Ag-NPs and Ag acetate was 9 mg/kg in this study. Ag content of all tissues investigated in this study increased comparing to negative control group after both Ag-NPs and Ag acetate administration. However, the Ag concentrations in tissues were found lower following exposure to Ag-NPs than those of Ag acetate. As explained by authors, this was because of a higher fecal excretion of Ag after administration of Ag-NPs comparing to Ag acetate. The excretion pattern of Ag was evaluated in this study by measuring the

amounts of Ag in feces and urine after 24 hours of collection. The excretion of Ag with urine was low whereas a high amount of Ag was excreted in feces. Authors suggested that differences between the fecal excretions of Ag in the Ag-NPs and Ag acetate administrated rats may be due to the enhanced binding of NPs to non-digestible food components as well as lower intestinal barrier passage of Ag-NPs than Ag acetate (165).

A study on toxicity and inflammatory responses of Ag-NPs in mice was conducted by Park et al. In this study, authors compared the toxicity of the different sized (22, 42, 71 and 323 nm) Ag particles (1 mg/kg) following 14 days repeated oral administration. According to results of tissue distribution of different sized Ag-NPs, small sized NPs were accumulated in brain, lung, liver, kidney and testis while Ag was not in detectable ranges in non-treated and large sized 323 nm treated groups. Also authors determined the adverse effects of Ag-NPs (42 nm) following repeated oral administration for 28 days in concentrations of 0.25, 0.5 and 1 mg/kg. The levels of AST, ALT and alkaline phosphatase (markers indicative for liver damage) were significantly increased in the group treated with highest dosage (1 mg/kg) in this study. Authors indicated that this result mean that Ag-NPs may induce hepatotoxicity by repeated oral administration. However, no histopathological evidence was observed in liver. On the other hand, inflammatory responses were found to be significantly induced by oral administration with increase of cytokine production, increased B cell distribution and inflammatory cell infiltrates (169).

These findings are in contrast with those of recent studies conducted by Zande et al. In this study the toxicokinetics and tissue distribution of two types of Ag-NPs (non-coated and PVP-coated) and of Ag ions ($AgNO_3$) in rats following a 28 days repeated oral exposure were investigated. No significant differences were found in ALT and AST plasma levels as well as antibody levels in blood, lymphocyte proliferation, cytokine release and natural killer cell activity in both Ag-NPs and $AgNO_3$ treated groups versus

control group, signifying that there is no indication of acute hepatotoxicity or immunotoxicity of the Ag exposure (166).

An important data in the Zande et al. study was the presence of Ag at high concentrations in brain even 2 months postexposure. This suggests that accumulated Ag in the brain can be expected to be long lasting (166). Some previous reports mentioned that Ag-NPs can cross the blood-brain barrier (BBB), cause damage to the barrier integrity and also alter sensory, motor and cognitive functions in mice or rat, depending on the dose and duration of exposure (170-172). For this reason, accumulation as well as staying of Ag in brain are alarming and should be considered in a risk assessment of Ag-NPs.

A subchronic (90-day) whole-body inhalation study (6 h/day, 5 days/week, for 13 weeks) of Ag-NPs (18–19 nm) in rats was conducted by Sung et al. The administrated NPs were at low (49 $\mu g/m^3$), medium (133 $\mu g/m^3$) and high (515 $\mu g/m^3$) doses. Histopathological examination indicated increases in lesions related to Ag-NPs exposure, including mixed inflammatory cell infiltrate, chronic alveolar inflammation, and small granulomatous lesions at all particle concentrations. The Ag concentration was observed to increase in the blood, indicating that Ag was transferred into the circulation from the lung. The tissue distribution of NPs was also investigated and main targets of particle accumulation and toxicity were observed to be the lungs and liver (173).

These findings are in contrast to those of Ji et al, who exposed rats (6 hours/day, 5 days/week, for 28 days) to Ag-NPs (<16 nm) at low (0.48 $\mu g/m^3$), medium (3.48 $\mu g/m^3$) and high (61 $\mu g/m^3$) concentrations via inhalation. No significant changes in the hematology and blood biochemical values were observed versus fresh air control group in this study. Furthermore, apart from two cases of hepatic focal necrosis among the male rats and one case among the female rats in the high-dose group, histopathological analysis did not show any distinct findings within other organs. It should be considered that the concentration of administered Ag-NPs and the exposure duration of this study were far below the Sung et al.

study and this may account for the different results obtained from these two studies (174).

Vlachou et al studied the safety and systemic absorption of Ag-NPs contained within one of commercially available burn wound dressings (Acticoat) following dermal exposure. A total of 30 burn patients were treated with Acticoat in this clinical study. Serum Ag levels were measured before, during, and at discontinuation of Acticoat treatment and again at 3 and 6 months following completion of treatment. Observations showed that the median maximum serum Ag level measured in this study was below the maximum level previously reported in the literature for people treated with Ag sulfadiazine cream. Serum levels returned to normal 6 months following the cessation of treatment. No biochemical or hematological indicators of toxicity were related to Ag absorption, and so the authors concluded that the Acticoat dressing is safe for use on burns (175). Although this study highlights the systemic transfer of Ag following topical administration, it still remain unknown that this may derive from Ag-NPs itself or the release of Ag^+ from NPs.

In line with these findings, Trop et al evaluated the safety of Acticoat in a case study. The liver enzyme levels were found to be high in patient, insinuating hepatotoxicity as a consequence of treatment. As soon as the local application was aborted, liver enzymes returned to the normal levels. As well as liver enzymes, the Ag levels in plasma and urine were clearly elevated during treatment period with Acticoat. Authors suggested that the possibility of toxic Ag effect in burn patients treated with Actiocat should be considered and Ag levels in plasma and/or urine should be monitored. In this study, it is unclear whether the symptoms were result of the Actiocat dressing or of the burn itself. Also again it is not clear that these findings are arising from contribution of Ag^+ or Ag-NPs (176).

In another study, Lankveld et al investigated the kinetics of the tissue distribution of Ag-NPs of different sizes (20, 80 and 110 nm) in rats up to 16 days after intravenous administration once daily for five consecutive days.

Results showed that Ag-NPs distributed rapidly to all organs following both single and repeated intravenous administration regardless of size. For 20 nm Ag-NPs most accumulation observed in liver followed by kidneys and spleen, while 80 and 100 nm particles mainly distributed to spleen followed by liver and lung. Ag-NPs distribution to other organs was seemed to be independent from particle size. Such size dependent distribution suggests size dependent toxicity and health risks. For all time points investigated in this study, the concentration of 20 nm Ag-NPs in liver, spleen and lung was remarkably lower than those of larger NPs. Authors explained that this finding may be due to the faster elimination/excretion, dissolution of Ag-NPs to Ag^{+} and redistribution of 20 nm particles to other organs (177).

Tiwari et al studied the effect of various doses (4, 10, 20, and 40 mg/kg) of Ag-NPs in rats after intravenously administration. Body weight, organ coefficient, whole blood count, and biochemistry panel assay for liver function enzyme, comet assay, ROS, and histological parameter were analyzed in this study. Significant changes were found in hematological parameters in the 20 and 40 mg/kg groups. Also in the 40 mg/kg group, a significant increase was found in liver function enzymes like ALT and AST. ROS in blood serum showed increment in the high dose group. Tail migration results in comet assay indicated damage in the DNA strand in the high dose group. Authors suggested that, the Ag-NP in doses below 10 mg/kg is safe for biomedical application and has no side-effects, but its high dose (> 20 mg/kg) is toxic (178).

Rahman et al investigated the expression of genes related to oxidative stress in different regions of the mouse brain after intraperitoneal injection of Ag-NPs. Results revealed the expression of genes associated with oxidative stress in mouse brain indicates Ag-NPs may produce neurotoxicity by stimulating oxidative stress generation and altered gene expression, leading to apoptosis (179). Although the finding achieved from this study is so valuable, there were no data measuring Ag content of the brain tissue and the administered doses (100, 500 and 1000 mg/kg) were excessively high.

Furthermore, the reality of exposure route should be considered by investigators, since the oral and inhalation exposures are more realistic than that of intraperitoneal or intravenous in risk assessments of NPs.

2.5.4. Reproductive and Developmental Toxicity of Ag-NPs

The sensitivity of reproductive and developmental outcomes to toxic substances causes significant public concern and for NPs this is even greater. As discussed previously, there are few studies investigating the toxic effects of Ag-NPs. However, there are even fewer studies containing data on detrimental consequences on fertility, pregnancy and development of offspring (180).

The mammalian female reproductive system is composed of hypothalamic-pituitary-ovarian axis and reproductive organs including the oviducts, uterus, vagina and external genitalia. This system's normal operation depends on the perfect positive and negative feedback between various components of the axis (Figure 2.7). The interference of xenobiotics with the female reproductive system may disrupt this normal operation (181).

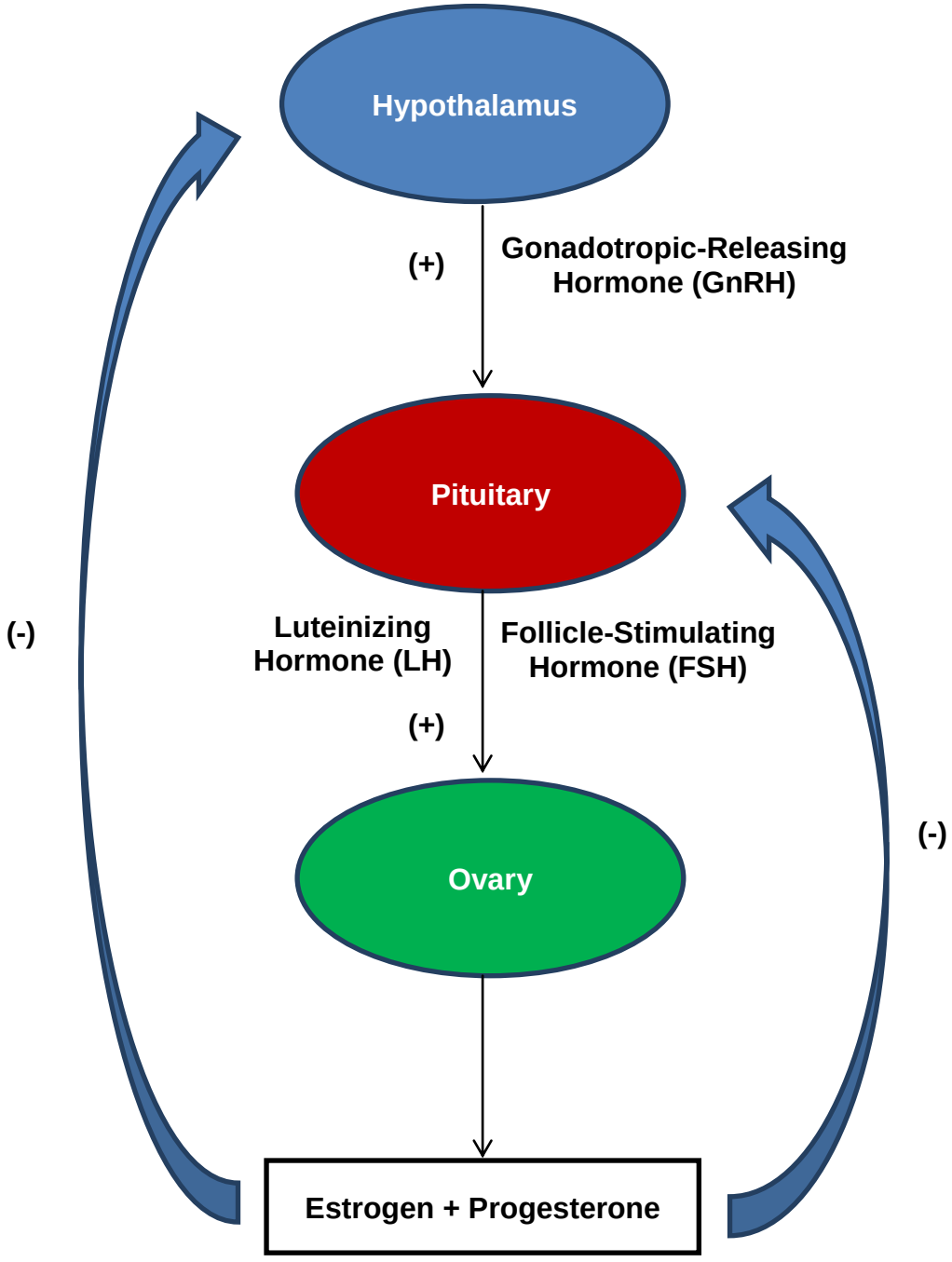


Figure 2.7. The hypothalamic-pituitary-ovarian axis of the female reproductive system. (+) represents positive feedback, and (-) represents negative feedback (181).

In a study conducted by Lee et al, the impact of Ag-NPs on zebrafish embryo development was investigated. Results showed that Ag-NPs can passively diffuse into developing embryos and create abnormalities on embryonic development in a dose-dependent manner (182).

In another study, Philbrook et al investigated the effects of orally administered Ag-NPs on reproduction and development in two distinct models.

In this study, pregnant mice were received a single dose of 10, 100, 1000 mg/kg Ag-NPs or the vehicle control by oral gavage on gestational day (GD) 9. On GD 19, one day prior to spontaneous delivery, animals were sacrificed and fetuses were collected for further studies. Results indicated no obvious signs of maternal toxicity, including behavioral changes or weight loss throughout 10 days post-exposure in dams treated with any of the three doses of Ag-NPs. However, a significant increase was observed in the number of non-viable fetuses obtained from dams exposed to 10 mg/kg Ag-NPs. At higher concentrations, this effect was not apparent. Authors argue that this finding may be due to facilitating of aggregates caused by higher gut concentrations resulting in prevented internalization via the gastrointestinal tract and reduced toxicity on fetuses at high doses. TEM analysis identified the presence of Ag-NPs in the fetal liver and kidneys. However, histological analysis of these organs showed no occurrence of cell death or inflammation.

Another model used in this study was *Drosophila melanogaster*. Results revealed a significant decrease in developmental success when *Drosophila* were treated with five different concentrations of Ag-NPs (0.005–0.5%). Insects such as *Drosophila*, lay eggs that undergo metamorphosis that define distinct developmental stages, but mice undergo through an internal development stage. Due to this and other distinct differences in the reproductive physiology of vertebrates and invertebrates, the same toxic effects on reproductive success in both models suggest cause for concern for a broad range of metazoans (183).

Li et al, revealed that Ag-NP exerts cytotoxic effects on mouse blastocysts but not as strong as that of the Ag^{+} ion. Also the implantation success rate of blastocysts pretreated with Ag-NPs was found lower than that of control in this study. In addition, pretreatment with Ag-NPs induced a high resorption rate of post-implantation embryos and a decrease in fetal weight (184).

In another study, exposure of nematode, *Caenorhabditis elegans*, to Ag-NPs resulted in significant decrease in reproduction ability with the increased ROS formation and expression of genes related to oxidative stress (185).

In a recent study, Hong et al conducted a combined repeated-dose toxicity study of citrate-capped Ag-NPs with reproduction and developmental toxicity in rats orally treated with 62.5, 125 and 250 mg/kg, once a day for 42 days for males and up to 52 days for females.

The results of tissue distribution of Ag in mother rats showed 34-fold increase in liver of Ag-NPs treated rats compared to the level in control livers. The Ag levels in lung and kidney were also significantly increased. Especially, the level of Ag in the lungs of treated rats was markedly increased compared to the level in liver or kidney. However, Ag-NPs did not cause any significant changes in the hematology, serum biochemistry, and histopathology and toxicity endpoints of reproduction /developmental screening test including mating, fertility, implantation, delivery and fetus (186).

The possible Ag distribution in offspring tissues as well as oxidative stress parameters as a known mechanism of Ag-NPs was not investigated in this study.

More recently, Yu et al investigated the potential effects of Ag-NPs on pregnant dams and embryo-fetal development in rats. Ag-NPs were administered to pregnant rats by oral gavage at concentrations of 0, 100, 300 and 1000 mg/kg/day in this study. The results showed that treatment with Ag-

NPs cause a decrease in catalase and glutathione reductase activities as well as reduction in glutathione content in dams liver tissue. However, no treatment related changes in maternal body weight, food consumption, gross findings, serum biochemistry, organ weight, gestation index, fetal deaths, fetal and placental weights, sex ratio and morphological alterations were observed between the groups (187).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Silver nitrate	Sigma
tri-Sodium citrate dihydrate	Merck
Nitric acid	Sigma
Ag standard	Fluka
Palladium (Pd) matrix modifier solution	Sigma
Magnesium (Mg) matrix modifier solution	Fluka
Potassium chloride	Riedel-deHaen
Sodium carbonate	Riedel-deHaen
Sodium hydroxide	Riedel-deHaen
Copper (II) sulfate-5-hydrate	Riedel-deHaen
Potassium sodium tartrate tetrahydrate	Riedel-deHaen
Bovine serum albumin (BSA)	Sigma
Folin & Ciocalteu's phenol reagent	Sigma
Sodium phosphate monobasic	Riedel-deHaen
di-Sodium hydrogen phosphate dihydrate	Fluka
Xanthine sodium	Sigma
2-(4—iodophenyl)-3-(4-nitrophenol)-5 phenyltetrazolium chloride (INT)	Sigma
3-(Cyclohexylamino)-1-propane sulfonic acid (CAPS)	Sigma
Ethylene diamine tetra-acetic acid disodium salt dehydrate (EDTA)	Sigma
xanthine oxidase	Roche Diagnostics GmbH
SOD from bovine erythrocytes	Sigma
Hydrogen peroxide (H_2O_2)	Merck
Catalase from bovine liver	Sigma
Trizma base	Sigma
GSH	Sigma

NADPH	Sigma
Sodium azide	Sigma
tert-Butyl hydroperoxide	Sigma
Trichloroacetic acid	Riedel-deHaen
2-Thiobarbituric acid (TBA)	Sigma
1,1,3,3-Tetramethoxypropane	Sigma
Dodecyl sulfate sodium salt (SDS)	Merck
Acetic acid	J.T.Baker
Nitrate reductase	Sigma
Sulfanilamide	Sigma
Phosphoric acid (H_3PO_4)	Merck
N-(1-naphtyl)ethylenediamine dihydrochloride	Sigma
Sodium nitrate	Fluka
Rat 3-NT enzyme-linked immunosorbent assay (ELISA) kit	Sunred biological technology company
Rat ALT ELISA kit	Uscn life science
Rat AST ELISA kit	Sunred biological technology company
Rat IL-6 ELISA kit	Sunred biological technology company
Rat progesterone ELISA kit	Sunred biological technology company
Rat estradiol ELISA kit	Sunred biological technology company
Rat oxytocin ELISA kit	MyBiosource
Rat MT-2 ELISA kit	Cusabio
Phosphate buffered saline	Lonza, BioWhittaker
Lymphoprep	Axis-Shield
RPMI-1640 Medium	Dipco
Agarose (high melting)	Sigma
Agarose (low melting)	Sigma
Sodium chloride (NaCl)	Sigma

Triton X-100	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Ethidium bromide	Sigma
Tryphan blue	Sigma
Formaldehyde	J.T.Baker
Haematoxylen	Bio optica
Eosin	DDK

3.1.2. Equipments

Doubly distilled deionized water Purification System	Milli-Q Millipore 18.2 MX/cm
Hot plate magnetic stirrer	Heidolph
Vortex	Heidolph, Reax top
Balance	Ohaus
Centrifuge	Sigma 3-16 PK
DLS	Malvan, Zetasizer NanoZS
UV-Vis spectrophotometer	Thermo, Evolution 300
TEM	Jeol, 2100 HR, operating at 200 kV
Sonicator	Sonorex RK 156 BH
Microwave digestion system	Mars 5, CEM
Atomic absorption spectrometry (AAS)	Analytic Jena, Zeenit 700
pH meter	Mettler Toledo, MP 220
UV/Vis microplate spectrophotometer	Multiskan Ascent, Thermo
Microplate auto washer	BioTek
Incubator	Heidolph, Unimax 1010
Electrophoresis	Bio-Rad
Cell counter and analyzer	Roche, Innovatis Codex XS
Light microscope	Zeiss, primo star
Fluorescent microscope	Zeiss
Light microscope	Olympus BX 51

Comet assay IV software	Version 4.3
Excelsior tissue processor	Thermo Shandon

3.2. Methods

3.2.1. Synthesis and Characterization of Ag-NPs

Synthesis of Ag-NPs

Ag-NPs capped with citrate were synthesized by citrate reduction method, which is known as Lee and Meisel method (94). In a summary, in this method, 90 mg of $AgNO_3$ was dissolved in 500 ml of deionized distilled water and heated to boiling using hot plate magnetic stirrer. Then 10 mL of trisodium citrate ($C_6H_5O_7Na_3$) solution (1 %) was added to the boiling solution dropwise. During this process solution was mixed vigorously and kept in darkness to avoid the influence of light. The color of the solution slowly turned yellowish, indicating the reduction of the silver ions. The solution was kept on boiling status for approximately 1 hour until the volume reached half of the initial volume (the final color was greenish). Then it was removed from the heating element and stirred until cooled down to room temperature. Mechanism of reaction could be expressed as follows:

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^{0} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2 \uparrow$$

This suspension was called 1X and then colloid was centrifuged at 5500 rpm, 60 minutes. After centrifugation, the water on top of the Ag-NPs precipitates were removed and replaced by distilled deionized water. This process was repeated two more times to ensure removal of excess molecules. Finally, the colloids were brought to final concentration of 50X by adding appropriate amount of water to Ag-NPs precipitates and re-dispersed by sonicating for 20 minutes.

All glass wares which were used in this process were prewashed with chromic acid and the remains of the acid were removed from the glasses with abundant amounts of deionized water.

During the study, in order to prevent any possibility of aggregation, Ag-NPs suspensions were prepared three times a week and suspensions were used up to a maximum of 2 days after preparation. The concentration of total Ag in Ag-NPs suspensions and $AgNO_3$ solutions were checked by AAS before administration.

Characterization of Ag-NPs

The size distribution and zeta potential measurements of the synthesized NPs were performed using zeta sizer Nano ZS at 25 °C. The nano ZS contains a 4mw He-Ne laser operating at a wavelength of 633 nm and an avalanche photodiode detector. The scattered light was detected at an angle of 173°. Furthermore, Ag-NPs were characterized UV-Vis spectroscopy and visualized by TEM.

3.2.2. Animals and Treatment

Male and female Sprague-Dawley rats were obtained from the Yeditepe University Medical School Experimental Research Center (YUDETAM) and housed at controlled room temperature (21±1°C) with 12:12 h light-dark cycle. The experimental protocol was approved by the Ethic Committee of Yeditepe University. The rats were acclimatized to the laboratory environment for 1 week before the initiation of the experiment. Standard pellet diet and water were provided *ad libitum*. For mating, two females were placed into a cage with a male rat overnight. Successful mating was confirmed by the presence of sperm in the vaginal smear. Upon confirmed mating, the pregnant dams were removed, weighed and housed in polypropylene cages with bedding in pairs. The day with positive mating evident was designated as day 0 of gestation (GD 0) and the day of spontaneous delivery was designed as postnatal day (PND) 1. The animals were assigned to five groups of 10 animals each and gavaged once daily at approximately same time from GD 7 to GD 20 with 0 (deionized water)[1], low dose (0.2 mg/kg), middle dose (2 mg/kg) and high dose (20 mg/kg) Ag-NPs suspension as well as $AgNO_3$ ([Ag] = 20 mg/kg). During the experiment period dams were weighed each day and dosages volumes were adjusted daily according to the body weight changes (dosing volumes were 4 ml/kg).

[1]4 animals in this group were treated two months before the other animals.

On GD 18, dams were housed separately and were monitored daily between 0900 h and 1800 h for birth. After parturition (PND 1), on PND 2, weights of dams and individual pups were recorded. The pups were counted, sexed and then dams as well as pups were sacrificed by decapitation. Brain, heart, lung, liver, kidneys, spleen, ovary, and uterus of dams as well as brain, lung, liver, kidneys and stomachs containing milk of pups from each litter dissected, weighed and frozen immediately on dry ice. All samples were stored at -80 ˚C until the analysis is conducted.

Trunk blood of dams and all pups within each litter was also collected in vacutainers containing Ethylene diamine tetraacetic acid (EDTA). Then they were centrifuged for 10 minutes at 4500 rpm and 4 ˚C within 1 hour after collection. After separation of plasma, the buffy coat was removed and the packed cells were washed three times with two volumes of isotonic saline. Then, a known volume of erythrocytes was lysed with cold distilled water (1:4), stored in a refrigerator at 4 ˚C for 15 minutes and the cell debris were removed by centrifugation (3200 rpm at 4 ˚C for 10 minutes). Erythrocyte lysates as well as plasma samples were divided into portions and stored at -80 ˚C until analysis is performed.

3.2.3. Determination of Ag in Tissue and Milk

Tissue samples of all dams and 2-3 pups of each litter as well as milk samples were digested with concentrated nitric acid by using a microwave digestion system.

The concentration of Ag in digested samples was determined by graphite furnace atomic absorption spectrometry with Zeeman background correction. The calibration curve was plotted with standard solution of Ag (3–20 ng/mL). Suitable dilutions were prepared from digested samples with triply distilled water. The manufacturer's application notes were used for metal measurements checking validation parameters. Table 3.1 shows atomic absorption spectrometer furnace parameters for Ag analysis.

Table 3.1. Atomic absorption spectrometer furnace parameters.

Wavelength (nm)	Maximum ash temperature (°C)	Atomization temperature (°C)	Modifier	Zeeman mode*
328.1	700	1600	Pd/Mg (NO_3)	3-field dynamic**

*The measurement mode for the background correction. **The measurement of the absorbance values at various magnetic field strengths.

3.2.4. Determination of ALT, AST and IL-6 Levels

The ALT, AST and IL-6 levels were measured by ELISA kits in plasma samples.

For ALT assay, standards or samples and a biotin conjugated antibody specific to ALT were added to the microtiter 96-well plate which has been pre-coated with an antibody specific to ALT. Next, avidin conjugated to horseradish peroxidase was added to each well and incubated. Then tetramethylbenzidine (TMB) substrate solution was added. TMB solution makes the reactions very easy to monitor, since in its reduced form, TMB is colorless and in oxidized form is blue. Finally, the enzyme substrate reaction is terminated by the addition of sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The concentration of ALT in the samples was then determined by comparing the OD of the samples to the standard curve. The results were expressed as ng/mL.

AST and IL-6 levels in plasma were determined by ELISA kits with the same principle. Briefly, 50 µL of standard solutions and 40 µL of samples added to well plates which have been pre-coated with appropriate monoclonal antibody. Then, 10 µL of related antibody as well as 50 µL of streptavidin conjugated horseradish peroxidase were added to wells and incubated 60 minutes at 37 ˚C. At the end of this period, plates were washed and drained five times with washing solution and 50 µL of chromogen

solutions were added to each wells. Next, plates were incubated for 10 minutes at 37 ˚C away from light. After incubation, 50 µL of stop solution was added into each well to stop the reaction. The blue color changed to yellow immediately at this stage. Finally, the absorbance was measured under 450 nm wavelength and the results were calculated by comparing the absorbance values of the samples to the standard curve. The results were expressed as U/L for AST and ng/L for IL-6.

3.2.5. Measurement of Oxidative Stress Parameters

Sample Preparation

Brain and liver samples were minced and homogenized with a glass homogenizer in a cold potassium chloride solution (1.15%) and centrifuged at 4.500 rpm and 4 ˚C for 15 minutes. The obtained supernatants were then used to analyze MDA, SOD, CAT and GPx. These parameters were also measured in erythrocyte part of blood samples which prepared as described previously. In addition to above oxidative stress parameters, 3-NT was also determined in plasma samples.

Determination of Protein Content in Samples

The protein content in erythrocyte lysate or brain and liver homogenates was determined according to the Lowry method (188). Briefly, main solutions including reagent A (2 % sodium carbonate in 0.1 N sodium hydroxide), reagent B (0.5 % copper sulfate) and reagent C (1 % potassium sodium tartrate) were prepared. Then 50 mL of reagent A, 0.5 mL of reagent B and 1 mL of reagent C were mixed together to obtain alkaline copper reagent. Working standard solutions were prepared from BSA in concentration range of 0.625-20 mg/mL.

For protein content measurement purposes, 0.01 mL of standard solutions or samples were mixed with 1 mL distilled water and 4.5 mL alkaline copper reagent and allowed to stand for 10 minutes at room temperature. Then 0.5 mL of Folin’s reagent (1:2 v/v) was added to this

solution, mixed and allowed to stand for 20 minutes at room temperature. Finally, the optical density (OD) of standard and sample solutions was measured under 640 nm wavelength.

Determination of the activities of antioxidant enzymes

SOD activity was measured according to previously described method by Aydin et al (189) and was expressed as kU/g protein. Shortly, each hemolysate or tissue homogenate was diluted 1:400 and 1:40 with 10 mM phosphate buffer (pH 7.0) respectively. 25 µL of diluted sample was then mixed with 850 µL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L INT in a buffer solution containing 50 mmol/L CAPS and 0.94 mmol/L EDTA disodium dehydrate (pH 10.2). Then, 125 µL of xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 minutes against air. Standard solutions were prepared from stock solution of SOD from bovine erythrocytes in the concentration range between 0.217 and 5.2 kU/L and phosphate buffer was used as a blank solution.

For determination of the CAT activity, the reaction mixture was 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and erythrocyte lysate or tissue homogenate sample. The reduction rate of H_2O_2 was followed at 240 nm for 45 seconds against air at room temperature (190). CAT activity was expressed as kU/g protein. Phosphate buffer was used as a blank solution and the calibration curve was plotted with standard solutions (1.25-15 U/mL) prepared from CAT stock lyophilized powder.

In order to measure the GPx activity the reaction mixture was composed of 50 mmol/L tris buffer (pH 7.6) containing 1 mmol/L of EDTA disodium dihydrate, 2 mmol/L of GSH, 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide and 1000 U of GR. The reaction mixture (990 µL) and 10 µL of erythrocyte lysate (diluted 15 times) or tissue homogenate (diluted 17 times) were pipetted into a quartz cuvette and incubated for 5 minutes at room temperature. Then, the reaction was initiated with the addition of 10 µL of

tert-butyl hydroperoxide solution (1:1000). The decrease in NADPH absorbance was followed at 340 nm against air for 3 minutes and the difference in absorbance per minute was calculated. GPx activity was expressed as kU/g protein (189, 190).

Determination of Lipid Peroxidation Levels

The MDA levels, as an end-product and a reliable sign of lipid peroxidation, were determined in erythrocyte lysate samples according to the method described by Aydin et al (189). Samples or standard substance were mixed with phosphate buffer and trichloroacetic acid in 1.5 mL Eppendorf tubes and kept on ice bags in refrigerator (4 ˚C) for 2 hours. At the end of the period, the tubes were centrifuged at 4400 rpm and 4 ˚C for 10 minutes. Then, 500 µL of the supernatant was mixed with 38 µL of EDTA and 125 µL of TBA. The mixtures were vortexed and kept on boiling water bath for 15 minutes. The absorbance was recorded at 532 nm.

The MDA levels in the liver and brain homogenates were determined according to the method described by Jamall and Smith (191). Two hundred microliters of sample or standard substance, 200 µL of SDS (8.1 %), 1500 µL of acetic acid solution (20 %) and 1500 µL of TBA solution (0.8 %) were mixed. The volume was completed to 4000 µL with distilled water. The tubes were kept at 95 ˚C for 1 hour. Then, they were cooled under tap water and 2000 µL of the mixture was added to 2000 µL of trichloroacetic acid. They were centrifuged at 2300 rpm for 10 minutes. The absorbance of the supernatant was read at 532 nm.

Tetramethoxy propane solution was used as standard for plotting the calibration curve. The results were expressed as nmol/g protein.

Determination of NO_2^-/NO_3^- and 3-NT Levels

Plasma nitrite/nitrate levels were measured by using the Griess reaction according to Tracey et al method (192). The reaction mixture consisted of reduced NADPH, nitrate reductase and phosphate buffer (pH 7.6). After incubation of plasma samples or standard substance with reaction mixture for one hour at room temperature, Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% N-(1-naphtyl)ethylenediamine) was added to the samples. After incubating for 10 minutes, the absorbance was measured at 540 nm. Sodium nitrate solution in the concentration ranging from 0.0625 nmol/µL to 2 nmol/µL was used as standard for plotting the calibration curve. The results were expressed as nmol/mL.

3-NT plasma levels were assayed by a double antibody sandwich ELISA kit, using 96-well plates coated with monoclonal antibody specific for 3-NT. Biotinylated detection antibody and streptavidin conjugated horseradish peroxidase were used for detection of captured 3-NT. Then captured 3-NT was visualized using chromogen solutions. Absorbance readings were made at 450 nm, using a UV/Vis microplate spectrophotometer. 3-NT levels in plasma samples were then determined by interpolation from a standard curve. The results were expressed as nmol/mL.

3.2.6. Determination of MT-2 Levels

MT-2, as a main isoform of MT proteins, was measured in plasma samples of dams and offspring by a commercial ELISA kit. Briefly, standards and samples were pipetted into the walls which were pre-coated with antibody specific for MT-2 and incubated for 2 hours at 37 ˚C. At the end of this period, the liquid of each well was removed and the biotin conjugated antibody specific for MT-2 was added to each well. After incubation for 1 hour at 37 ˚C, each well was aspirated and washed three times by automated microplate washer. Then avidin conjugated horseradish peroxidase was added to each well and incubated for 1 hour. At the end of incubation period, wells were aspirated and washed five times. Following a wash to remove any

unbounded avidin-enzyme reagent, a substrate solution was added to the wells and color was developed in proportion to the amount of MT-2 bound in the initial step. Then the color development was stopped and the intensity of the color was measured at 450 nm. The results were expressed as ng/mL.

3.2.7. Determination of Progesterone, Estradiol and Oxytocin Levels

Commercial ELISA kits were used to assay the levels of progesterone, estradiol and oxytocin in plasma samples of dams.

Progesterone and estradiol levels were determined by ELISA kits with the same principle. Shortly, samples and standard solutions were added to the wells which were pre-coated with rat progesterone or estradiol monoclonal antibody and incubated for 60 minutes at 37 ˚C. At the end of this period, Progesterone or estradiol antibodies labeled with biotin and streptavidin horseradish peroxidase were added to each well to form immune complex. Then, wells were incubated and washed to remove the uncombined enzyme. By adding the chromogen solutions, the color of the liquid were changed into blue and at the effect of stopping solution containing acid, the color finally became yellow. The intensity of color was measured at 450 nm. The results were expressed as ng/mL for progesterone and ng/L for estradiol.

For oxytocin determination, the standards or samples, an antibody specific for oxytocin and horseradish peroxidase conjugated oxytocin were added to the pre-coated microtiter plate wells with goat-anti-rabbit antibody. Then, the competitive inhibition reaction was launched between with horseradish peroxidase labeled oxytocin and unlabeled oxytocin with the antibody. Next, a substrate solution was added to the wells and the color was developed in apposite to the amount of oxytocin in the sample. Finally, the color development was stopped and the intensity of the color was measured at 450 nm. The results were expressed as pg/mL.

3.2.8. Comet Assay

Lymphocyte separation

Whole blood samples of dams which were collected formerly into EDTA tubes were diluted by the addition of equal volume of phosphate buffered saline (PBS). Then, the diluted blood layered over Lymphoprep (2:1) in a 12–15 mm centrifuge tube and Centrifuged at 1500 rpm for 5 minutes at 4 ˚C. After centrifugation the distinct band at the sample/medium interface were best removed using a Pasteur pipette and the cells were washed with RPMI-1640 medium. In order to determine cell viability 0.4% tryphan blue was used and the cells were counted with an automated cell counter/analyzer. As a positive control, lymphocytes were treated with 50 µM H_2O_2 and incubated for 5 minute on ice. After incubation, cell viability was again examined using tryphan blue.

Assay Procedure

The alkaline comet assay technique was performed as described in Sandal et al (193). Briefly, microscope slides were precoated with 0.75% (w/v) high melting agarose at PBS. The lymphocytes were mixed with 100 µl of 0.5% (w/v) low melting agarose at 37 ˚C and the cell suspension was layered onto precoated slides. The slides were immediately covered with a large cover slip and kept at 4 ˚C for 5 minutes to allow the agarose to solidify. After the cover slips were removed, the slides were immersed in ice cold freshly prepared lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Trizma base, pH 10) with 1% Triton X-100 and 10% DMSO for 1 hour.

Electrophoresis

After lysis, the slides were placed in a horizontal gel electrophoresis tank positioned close to the anode and covered with fresh alkaline electrophoresis buffer solution containing 1mM EDTA, 300mM NaOH (pH > 13) for 20 minutes at 4 ˚C. Electrophoresis was conducted at 25V (0.83 V/cm) and 300 mA for 20 minutes. After electrophoresis, the slides were washed

three times with neutralizing buffer (0.4M Trizma base, pH 7.5) at 4 ˚C for 5 minutes each. Then the slides were dried and stained with 20 µg/ml ethidium bromide and covered with a cover slip. The slides were incubated at 4 ˚C in a refrigerator for 20 minutes before scoring.

Slide Scoring

Fifty cells per slide and two slides per sample were scored to evaluate DNA damage. The slides were examined under a fluorescent microscope equipped with suitable filters at 200× magnification. The cells were scored using comet assay IV software according to the relative intensity of fluorescence in the tail. Tail intensity values represent the DNA damage.

3.2.9. Histopathology

The brain, lung, spleen, heart, kidney, uterus, ovaries and liver tissues from 5 dams in each group, and brain, heart, liver, lung and kidney of one pup from related litter were fixed in 10% formalin, for 24 hour. After routine tissue processing, the tissues were embedded in paraffin. 4µm thick sections obtained from each paraffin block were stained with haematoxylen and eosin for histopathological evaluation under light microscope.

3.2.10. Statistical Analysis

All analyses were performed using SPSS 21.0 (Statistical Packages of Social Sciences) program. The Kolmogorov–Smirnov test was used to test normal distribution assumption for continuous variables. Descriptive statistics for continuous variables were shown as mean ± standard deviation. All data were analyzed by the use of Levene test to determine homogeneity of variance. For comparing more than two groups of variables, data with normal distribution and homogeneity of variance were analyzed using ANOVA and data with normal distribution but heterogeneity of variance were analyzed by Welch test instead of ANOVA. For pair wise comparisons, Tukey test was used for data with normal distribution and homogeneity of variance. Dunnett T3 test was used for data with normal distribution and heterogeneity of

variance. In cases where normal distribution could not be obtained, Kruskal-Wallis test was used for comparing more than two groups of variables. Mann-whitney U test was used for pairwise comparisons. Litter size was included as a covariate in analyses of body and absolute organ weights of the pups. To determine whether the relationship between the two variables Pearson correlation test was used. A $p < 0.05$ was deemed statistically significant in all experiments.

4. RESULTS

4.1. Characterization of Ag-NPs Suspension

DLS analysis revealed average particle diameter of 70 nm. The zeta potential of synthesized NPs was around -32 mV (Figure 4.1), indicating the electrostatic stabilization of NPs (194). As illustrated in Figure 4.2, the maximum absorption of the solution was recorded at ~420 nm on the UV/Vis spectrum, which is characteristic for the Ag-NPs in this range of diameter (195). These findings were also confirmed via TEM imaging (Figure 4.3).

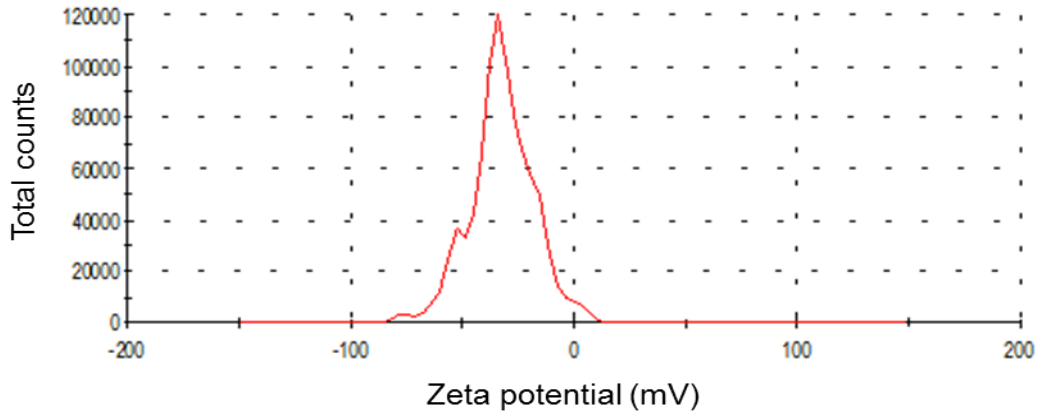


Figure 4.1. Zeta potential of synthesized Ag-NPs

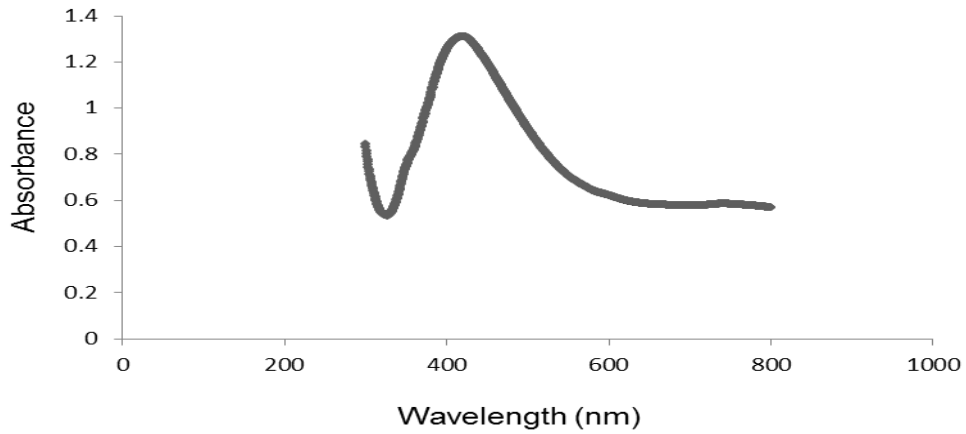


Figure 4.2. UV/Vis absorption spectrum of Ag-NPs obtained

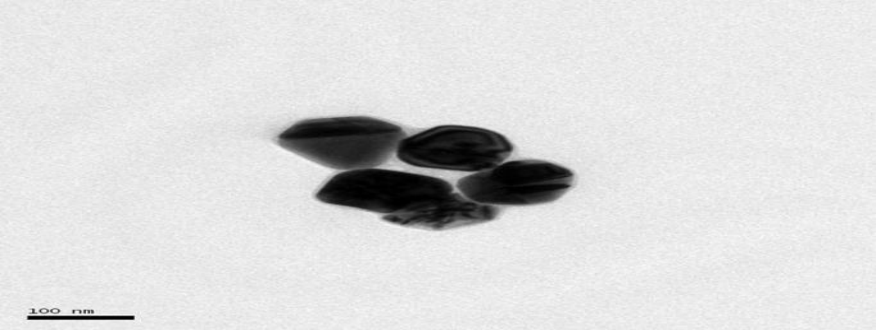


Figure 4.3. TEM image of Ag-NPs showing a range of sizes and shapes. The average particle diameter was 70 nm

4.2. Toxicological Evaluation of Ag in Dams and Pups

4.2.1. Maternal and Fetal Observations

There was no obvious indication of maternal toxicity during the experimental period and the dams' behavior appeared to be normal. No changes in general appearance of pups were observed within the exposed to Ag compared to the control groups (Table 4.1).

Table 4.1. Pregnancy outcome and litter data.

Dams and litters	Control	0.2 mg/kg Ag-NPs	2 mg/kg Ag-NPs	20 mg/kg Ag-NPs	20 mg/kg $AgNO_3$
No. of dams (litters)	n= 10 (9)	n= 10 (8)	n= 10 (10)	n= 10 (10)	n= 10 (9)
Body weight gain (g)	77.2 ± 7.8	83.8 ± 9.7	80.6 ± 9.2	84.9 ± 4.9	77.7 ± 6.8
Pregnancy length (day)	20.9 ± 0.8	21.0 ± 0.0	20.8 ± 0.4	21.1 ± 0.3	21.1 ± 0.8
No. of implantation sites	13.0 ± 2.1	13.1 ± 1.7	13.1 ± 1.4	14.6 ± 1.1	14.4 ± 2.3
No. of resorption	1.5 ± 1.1	1.1 ± 1.5	0.9 ± 0.9	1.6 ± 1.0	1.0 ± 0.8
Litter size	11.5 ± 2.8	11.7 ± 2.5	12.2 ± 1.5	13.0 ± 1.4	13.4 ± 2.4
Fetal body weight (g)	6.6 ± 1.3	5.9 ± 0.7	5.6 ± 0.8	5.4 ± 0.6	5.9 ± 1.3
% Viability	87.6 ± 9.0	89.0 ±14.1	93.1 ± 7.0	89.3 ± 7.2	92.8 ± 5.9
% males	51.1 ± 16.2	52.8 ± 9.4	49.6 ± 13.2	56.6 ± 5.3	53.4 ± 14.1

Results were expressed as the mean ± standard deviation (SD)

There were no statistically significant differences in the pregnancy length and maternal body weight gain from GD 7 to GD 20. The number of implantation sites, litter size, fetal weight, fetal resorptions, percent of viability and gender distribution were similar between the groups ($p > 0.05$).

4.2.2. Organ Weights of Dams

Relative (organ weight / terminal body weight) and absolute organ weights of dams were represented in Table 4.2 and 4.3 respectively.

Table 4.2. Relative organ weights (% of body weight) of dams.

	Dose levels (mg/kg)				
	Control (n = 9)	0.2 Ag-NPs (n = 8)	2 Ag-NPs (n =10)	20 Ag-NPs (n = 10)	20 $AgNO_3$ (n = 9)
Brain	0.87 ± 0.10	0.87 ± 0.09	0.93 ± 0.06	[a,b]0.98 ± 0.06	0.95 ± 0.05
Lung	0.46 ± 0.05	0.52 ± 0.09	0.58 ± 0.16	0.52 ± 0.06	0.61 ± 0.16
Heart	0.34 ± 0.02	0.36 ± 0.08	0.36 ± 0.03	[a]0.39 ± 0.04	0.38 ± 0.03
Liver	3.82 ± 0.27	3.97 ± 0.31	3.77 ± 0.33	3.78 ± 0.28	4.06 ± 0.30
Spleen	0.21 ± 0.03	0.20 ± 0.02	0.19 ± 0.02	0.21 ± 0.04	0.21 ± 0.03
Kidney (Right)	0.39 ± 0.04	0.38 ± 0.02	0.37 ± 0.04	0.41 ± 0.06	0.42 ± 0.03
Kidney (Left)	0.38 ± 0.04	0.37 ± 0.03	0.37 ± 0.03	0.41 ± 0.03	0.42 ± 0.04
Ovary (Right)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Ovary (Left)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.01
Uterus	0.77 ± 0.24	1.01 ± 0.30	1.07 ± 0.16	[a]1.14 ± 0.20	0.99 ± 0.40

Results were expressed as the mean ± SD. [a] Significantly different from the control ($p < 0.05$), [b] significantly different from low dose ($p < 0.05$).

The relative weights of the heart and uterus were significantly higher at daily dose of 20 mg/kg Ag-NPs treated group comparing to the control group ($p < 0.05$). Also an increase in relative brain weight was observed in Ag exposed dams. This increase was statistically significant at 20 mg/kg/day Ag-NPs treated group comparing to low dose and control groups ($p < 0.05$). No significant changes in the relative weight of other organs were found (Table 4.2).

Table 4.3. Absolute organ weights (g) of dams.

	Dose levels (mg/kg)				
	Control (n = 9)	0.2 Ag-NPs (n = 8)	2 Ag-NPs (n= 10)	20 Ag-NPs (n=10)	20 $AgNO_3$ (n = 9)
Brain	1.86 ± 0.07	1.83 ± 0.05	1.87 ± 0.08	1.89 ± 0.07	1.90 ± 0.04
Lung	0.99 ± 0.07	1.08 ± 0.18	1.06 ± 0.19	1.01 ± 0.14	1.22 ± 0.26
Heart	0.73 ± 0.11	0.73 ± 0.06	0.72 ± 0.06	0.77 ± 0.11	0.76 ± 0.09
Liver	8.31 ± 1.25	8.36 ± 1.06	7.56 ± 0.99	7.36 ± 0.83	8.13 ± 0.72
Spleen	0.45 ± 0.09	0.41 ± 0.06	0.38 ± 0.05	0.40 ± 0.09	0.42 ± 0.07
Kidney (Right)	0.84 ± 0.08	0.80 ± 0.07	0.75 ± 0.07	0.81 ± 0.05	0.83 ± 0.09
Kidney (Left)	0.82 ± 0.09	0.78 ± 0.07	0.74 ± 0.06	0.79 ± 0.04	0.83 ± 0.11
Ovary (Right)	0.07 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01
Ovary (Left)	0.06 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
Uterus	1.62 ± 0.38	2.21 ± 0.49	2.15 ± 0.33[a]	2.20 ± 0.45	1.95 ± 0.73

[a] Significantly different from the control ($p < 0.05$). Results were expressed as the mean ± SD.

Absolute uterus weights were found higher than control group in all Ag treated groups. However, this increase was statistically significant just at middle dose Ag-NPs group ($p < 0.05$). No significant differences were found in absolute organ weights of other organs (Table 4.3).

4.2.3. Organ Weights of Pups

Relative and absolute organ weights of pups were represented in Table 4.4 and 4.5 respectively. No statistically significant changes were found in either relative or absolute organ weights of pups.

Table 4.4. Relative organ weights (% of body weight) of pups.

	Dose levels (mg/kg)				
	Control	0.2 Ag-NPs	2 Ag-NPs	20 Ag-NPs	20 $AgNO_3$
No of litters	9	8	10	10	9
Brain	4.62 ± 0.64	4.54 ± 0.51	4.71 ± 0.41	5.11 ± 0.45	4.90 ± 0.42
Lung	1.96 ± 0.13	2.08 ± 0.17	2.19 ± 0.22	2.22 ± 0.24	2.21 ± 0.23
Liver	4.05 ± 0.42	4.50 ± 0.44	4.44 ± 0.23	4.51 ± 0.54	4.36 ± 0.32
Kidneys	1.18 ± 0.16	1.11 ± 0.07	1.13 ± 0.06	1.25 ± 0.13	1.17 ± 0.05

Results were expressed as the mean of 6 pups in each litter ± SD.

Table 4.5. Absolute organ weights (g) of pups.

	Dose levels (mg/kg)				
	Control	0.2 Ag-NPs	2 Ag-NPs	20 Ag-NPs	20 $AgNO_3$
No of litters	9	8	10	10	9
Brain	0.31 ± 0.03	0.28 ± 0.01	0.27 ± 0.02	0.28 ± 0.03	0.30 ± 0.05
Lung	0.14 ± 0.02	0.13 ± 0.02	0.13 ± 0.01	0.12 ± 0.02	0.14 ± 0.03
Liver	0.28 ± 0.05	0.28 ± 0.04	0.26 ± 0.03	0.25 ± 0.02	0.26 ± 0.05
Kidneys	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.02

Results were expressed as the mean of 6 pups in each litter ± SD.

4.2.4. Ag Distribution in Dam's Tissues

Ag levels in tissue samples of dams were shown in Table 4.6 and Figure 4.4. In all Ag-NPs treated groups, Ag concentrations were higher than control group in all evaluated maternal tissues except for the heart, lung and

uterus in low dose NP treated dams. However, this increment was just statistically significant for the kidney, spleen and uterus ($p < 0.05$).

In all investigated tissues, Ag level was much higher in the $AgNO_3$ group in comparison to the Ag-NPs groups. In the heart, Ag level in $AgNO_3$ treated group was found significantly ($p < 0.001$) higher than control as well as low, middle and high dose Ag-NPs treated groups. Significant increment ($p < 0.05$) was also found in Ag level of uterus in $AgNO_3$ treated group *versus* low dose Ag-NPs and control groups as well as that of $AgNO_3$ group comparing to control ($p < 0.05$) in spleen. Similarly, brain tissue from the group exposed to $AgNO_3$ revealed a statistically significant increase in the Ag level as compared with middle ($p < 0.05$) and low dose Ag-NPs treated groups as well as control group ($p < 0.01$).

Table 4.6. Tissue content of Ag in dams (mean ± SD).

	Dose levels (mg/kg)				
Silver (µg/g tissue)	Control (n = 9)	0.2 Ag-NPs (n = 8)	2 Ag-NPs (n = 10)	20 Ag-NPs (n = 10)	20 $AgNO_3$ (n = 9)
Spleen	0.09 ± 0.08	0.42 ± 0.33	0.35 ± 0.18[a]	0.84 ± 0.76	2.8 ± 1.43[a]
Kidney	0.33 ± 0.24	0.61 ± 0.34	1.37 ± 0.69	1.79 ± 0.72[a, b]	4.96 ± 3.22
Liver	0.46 ± 0.25	0.82 ± 0.57	0.83 ± 0.68	1.04 ± 0.49	1.27 ± 0.65
Uterus	0.34 ± 0.30	0.29 ± 0.17	0.58 ± 0.25	0.89 ± 0.10[a,b]	1.71 ± 1.00[a,b]
Heart	0.34 ± 0.28	0.31 ± 0.21	0.76 ± 0.68	0.88 ± 0.70	4.97 ± 0.78[a,b,c,d]
Lung	0.36 ± 0.16	0.31 ± 0.25	0.43 ± 0.39	0.66 ± 0.61	0.70 ± 0.34
Brain	0.17 ± 0.13	0.20 ± 0.09	0.24 ± 0.06	0.29 ± 0.09	0.42 ± 0.21[a,b,c]
Plasma	< LOD	0.01 ± 0.01	0.08 ± 0.02[b]	0.17 ± 0.05[b,c]	0.67 ± 0.30[b,c,d]
Erythrocyte	< LOD	< LOD	0.01 ± 0.00	0.02 ± 0.01[c]	0.08 ± 0.02[c,d]

a: significantly different from control ($p < 0.05$), b: significantly different from low dose ($p < 0.05$), c: significantly different from middle dose ($p < 0.05$), d: significantly different from high dose ($p < 0.05$).

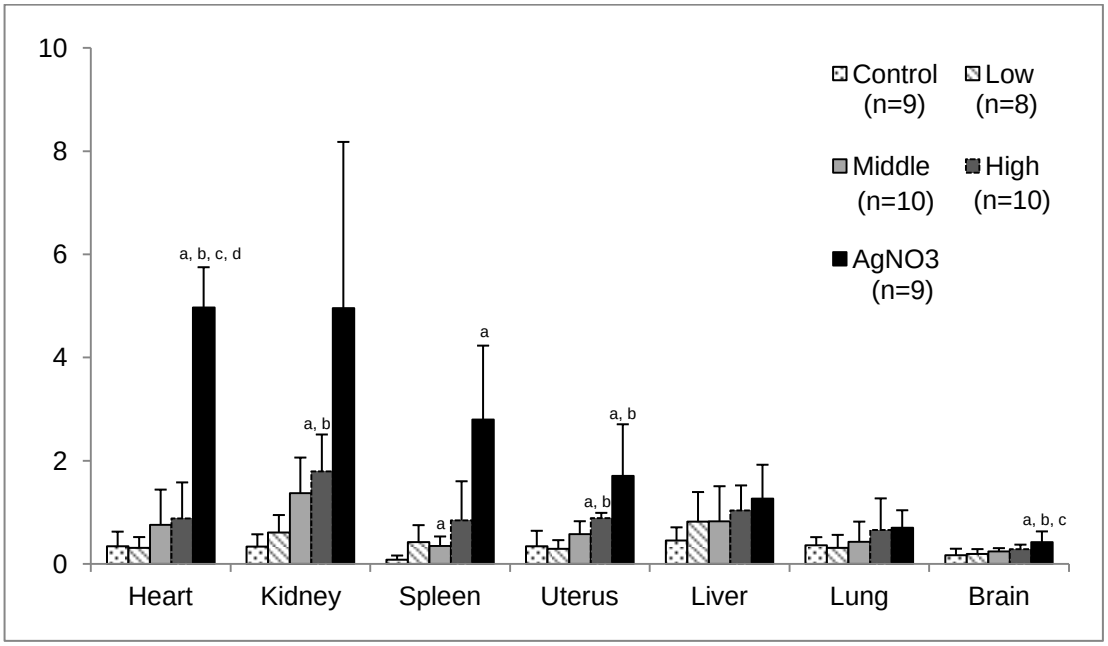


Figure 4.4. Ag content in dam's tissues (µg/g tissue). Results were expressed as the mean ± SD. a: significantly different from control ($p < 0.05$), b: significantly different from low dose ($p < 0.05$), c: significantly different from middle dose ($p < 0.05$), d: significantly different from high dose ($p < 0.05$).

The Ag concentration in erythrocyte and plasma samples of dams was also measured and given in Table 4.6 and Figure 4.5. Ag levels in erythrocyte at low dose Ag-NPs treated and control groups as well as control group in plasma were below the limit of determination (1.5 ng/ml), and are therefore not shown in the Figure.

Ag concentrations were significantly increased dose dependently in both erythrocyte ($p < 0.01$) and plasma ($p < 0.05$) parts of blood in Ag-NPs treated dams. Similar to other tissues, Ag concentration was much higher in the $AgNO_3$ group in comparison to the Ag-NPs groups.

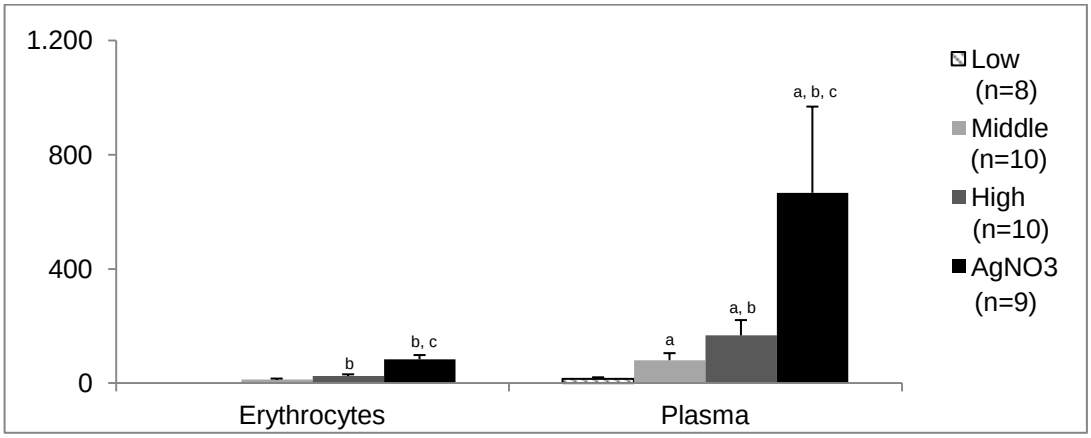


Figure 4.5. Ag content in dam's erythrocyte and plasma (ng/mL tissue). Results were expressed as the mean ± SD. a: significantly different from low dose ($p < 0.05$), b: significantly different from middle dose ($p < 0.05$), c: significantly different from high dose ($p < 0.05$).

4.2.5. Ag Distribution in Offspring's Tissues

Ag levels in brain, lung, liver and kidney of pups were represented in Table 4.7 and Figure 4.6.

Table 4.7. Tissue content of Ag in pups (mean ± SD).

*Silver (µg/g tissue)	Dose levels (mg/kg)				
	Control (**n = 9)	0.2 Ag-NPs (n = 8)	2 Ag-NPs (n = 10)	20 Ag-NPs (n = 10)	20 $AgNO_3$ (n = 9)
Kidney	0.52 ± 0.37	3.06 ± 0.85[a]	3.19 ± 0.73[a]	3.05 ± 1.45[a]	1.44 ± 0.89
Lung	0.72 ± 0.53	2.84 ± 1.94	2.09 ± 1.35	3.07 ± 1.60	2.34 ± 0.67[a]
Liver	0.27 ± 0.14	0.58 ± 0.21	0.47 ± 0.52	0.88 ± 0.55	0.62 ± 0.58
Brain	0.13 ± 0.04	0.20 ± 0.13	0.26 ± 0.09	0.26 ± 0.06	0.26 ± 0.01
Plasma	< LOD**	< LOD	< LOD	0.01 ± 0.00	0.04 ± 0.01
Erythrocyte	< LOD	< LOD	< LOD	0.02 ± 0.02	0.04 ± 0.03

*Results represent Ag content (µg/g) in 1 g of each litter. **n is composed from the average of three pups in each litter. a: significantly different from control ($p < 0.05$). **Limit of determination

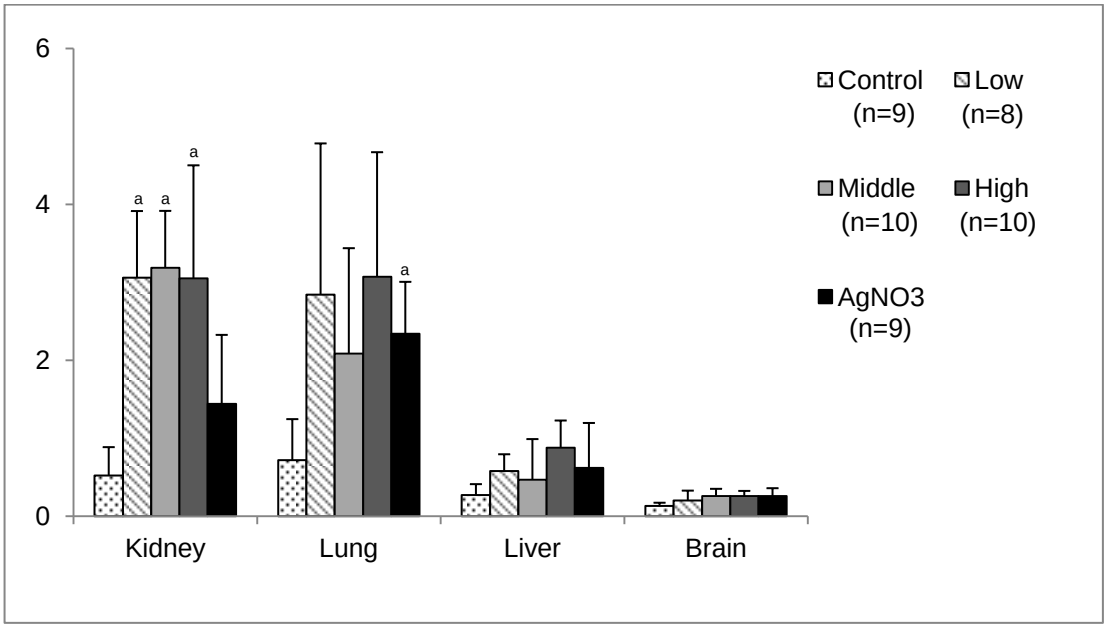


Figure 4.6. Ag content in pups tissues (µg/g tissue in 1 g of each litter). n is composed from the average of three pups in each litter. Results were expressed as the mean of three pups in each litter ± SD. a: significantly different from control ($p < 0.05$).

Ag level in high dose Ag-NPs treated group was 3.05 ± 1.45 µg/g in the kidney (5.8 fold versus to control group), 3.07 ± 1.60 µg/g in the lung (4.3 fold), 0.88 ± 0.35 µg/g in the liver (3.2 fold) and 0.26 ± 0.06 µg/g in the brain (2.0 fold).

Similar to dams, Ag content of all investigated tissues was found higher in Ag treated groups than control. However, only in kidney these increments were found statistically significant ($p < 0.01$). Also Ag level in lung tissue of $AgNO_3$ group was found significantly different from control ($p < 0.05$). Unlike to dams, Ag concentrations in $AgNO_3$ exposed group were similar or even lower than Ag-NPs treated group with the same dose level.

The Ag level in erythrocyte and plasma samples of pups was given in Table 4.7 and Figure 4.7. Ag levels in erythrocyte and plasma samples at middle and low dose Ag-NPs treated as well as control groups were below the limit of determination (1.5 ng/ml), and are therefore not shown in the

Figure. Similar to dams, Ag concentration in $AgNO_3$ group was found higher than Ag-NPs treated group with the same dose level. This increment was statistically significant ($p < 0.01$) in plasma.

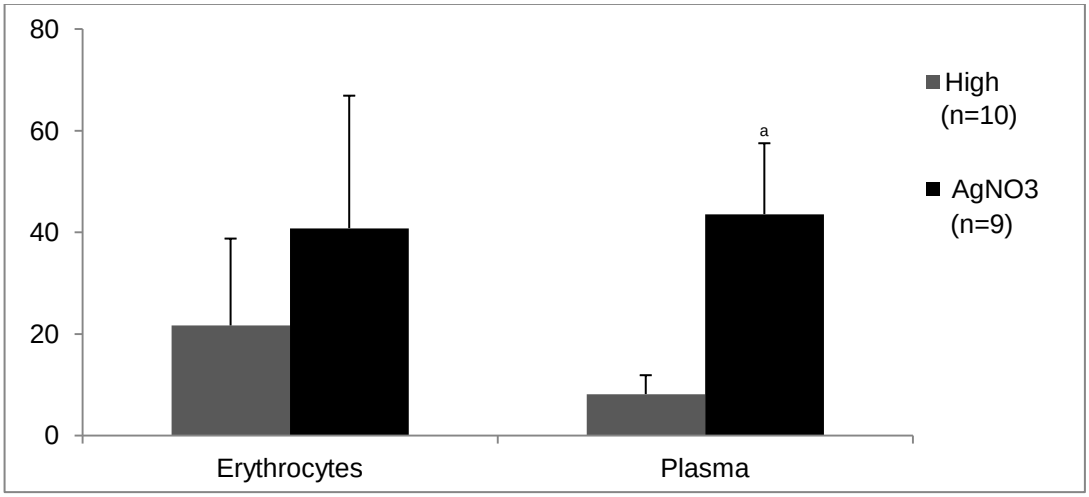


Figure 4.7. Ag content in pups erythrocyte and plasma (ng/mL tissue). Blood samples were pooled from all pups in each litter. Results were expressed as the mean ± SD. a: significantly different from high dose ($p < 0.05$).

Also milk samples were pooled from 4-5 pups' stomach in each litter and analyzed for Ag content. Although no statistically significant differences ($p = 0.19$) were found between groups, an enhancement in Ag content of treated groups was observed versus control (Table 4.8).

Table 4.8. Ag concentration in milk (mean ± SD).

Groups	Control	Low	Middle	High	$AgNO_3$
No. of litters (n)	n= 9	n= 8	n= 10	n= 10	n= 9
Ag content of milk (µg/g)	0.25 ± 0.21	0.33 ± 0.25	0.32 ± 0.11	0.66 ± 0.57	0.76 ± 0.45

Mean ± SD corresponding to the µg/g of milk in 1 g of each litter. Milk samples were pooled from 4-5 animals in each litter.

4.2.6. ALT, AST and IL-6 Levels in Dams and Pups

It was observed that the level of AST and IL-6 in dams was increased slightly in Ag treated groups compared to control. Similarly in pups, AST levels were increased in middle, high and $AgNO_3$ groups as well as all Ag exposed groups in IL-6 levels compared to control. However, these differences were not statistically significant ($p > 0.05$). The highest values for AST and IL-6 were obtained for $AgNO_3$ group in both dams and pups (Table 4.9). In addition, ALT levels were found to be same in all groups in either dams or pups.

Table 4.9. ALT, AST and IL-6 Levels in Dams and Pups.

	ALT (ng/mL)		AST (U/L)		IL-6 (ng/L)	
Groups	Dams	Pups	Dams	Pups	Dams	Pups
Control (n=9)	38.9 ± 18.3	16.2 ± 7.6	97.3 ± 15.8	59.9 ± 23.6	158.4 ± 12.1	94.2 ± 36.8
Low (n=8)	36.4 ± 14.7	18.6 ± 11.7	105.7 ± 22.6	55.6 ± 10.5	167.9 ± 17.4	108.6 ± 16.6
Middle (n=10)	42.3 ± 13.3	18.6 ± 4.5	113.8 ± 15.6	66.4 ± 12.9	173.3 ± 17.8	107.6 ± 36.1
High (n=10)	34.9 ± 10.4	16.8 ± 9.2	113.1 ± 13.8	70.9 ± 26.9	172.4 ± 19.3	117.7 ± 42.7
$AgNO_3$ (n=10)	39.3 ± 15.1	18.3 ± 7.1	123.2 ± 24.3	84.5 ± 31.5	180.6 ± 30.6	133.5 ± 44.2

Blood samples were pooled from all pups in each litter. Results were expressed as the mean ± SD.

4.2.7. Antioxidant Enzyme Activities in Dams and Pups

SOD

SOD activities in erythrocyte, liver and brain homogenates of dams and pups were represented in Figure 4.8 and 4.9 respectively.

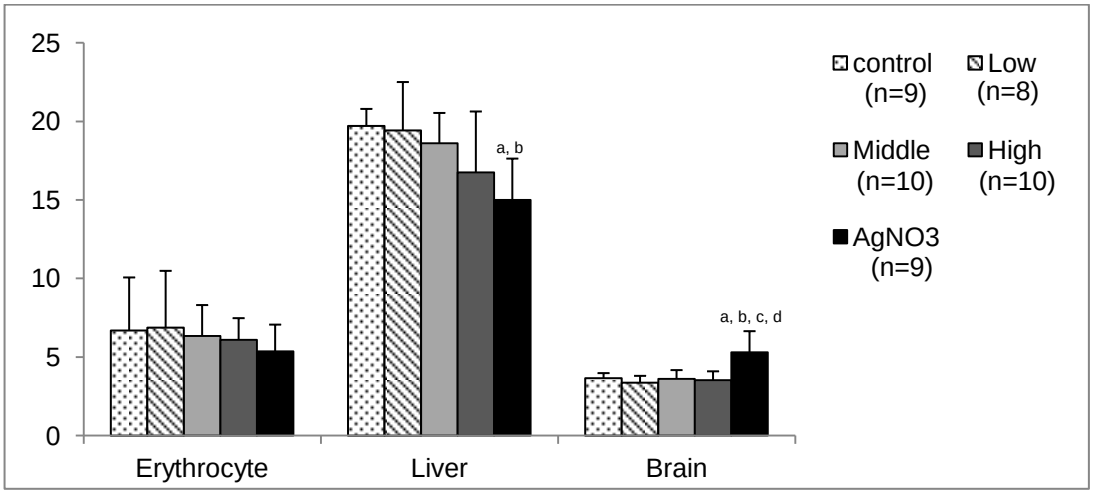


Figure 4.8. SOD activities of erythrocyte, liver and brain homogenates of dams (kU/g protein). The results were expressed as the mean of duplicates ± SD. a: significantly different from control ($p < 0.05$), b: significantly different from low dose ($p < 0.05$), c: significantly different from middle dose ($p < 0.05$), d: significantly different from high dose ($p < 0.05$).

No statistically significant change was found in Ag-NPs administered groups in dams. However, $AgNO_3$ group showed significant decrease in SOD levels comparing to low dose Ag-NPs treated and control groups in the liver homogenates ($p < 0.05$). Also, SOD levels in the brain homogenates of $AgNO_3$ group were significantly increased versus to all NP treated and control groups ($p < 0.05$).

SOD activities in erythrocyte as well as liver and brain homogenates in offspring were illustrated in Figure 4.9. No statistically significant differences was observed between groups ($p > 0.05$).

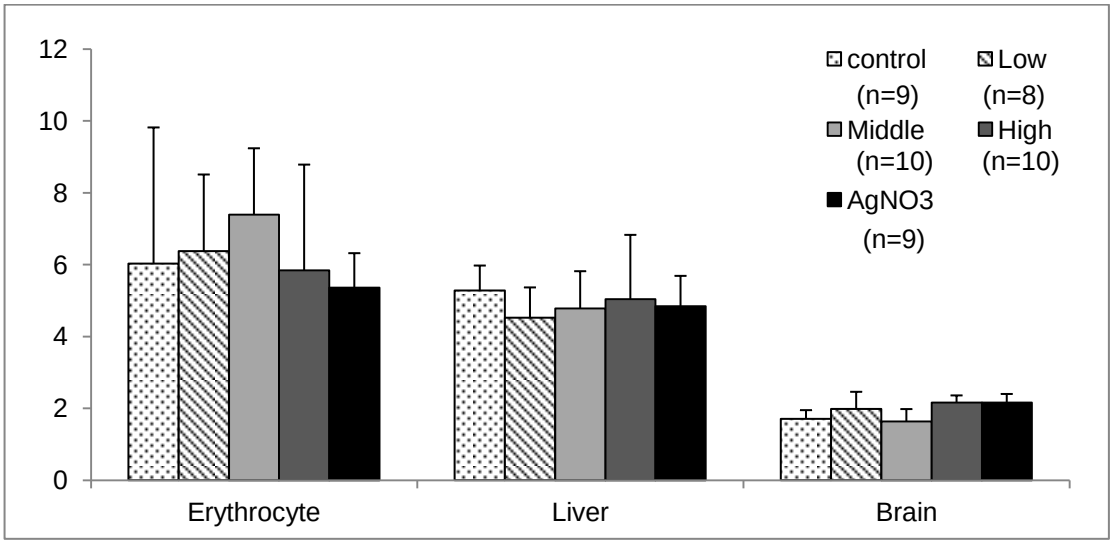


Figure 4.9. SOD activities of erythrocyte, liver and brain homogenates of pups (kU/g protein). n is composed from the average of three pups in each litter for pups' liver and brain. Blood samples were pooled from all pups in each litter. The results were expressed as the mean of three pups in each litter ± SD.

CAT

CAT activities of erythrocyte, liver and brain homogenates of dams and pups were summarized in Table 4.10, 4.11 and 4.12, respectively. No statistically significant change was observed in either dams or pups ($p > 0.05$).

Table 4.10. CAT activities in erythrocyte of dams and pups.

Groups	No. of dams (litters)	CAT (kU/g protein)	
		Dams	Pups
Control	9	239.1 ± 64.8	228.7 ± 131.6
Low	8	256.8 ± 138.9	349.4 ± 100.4
Middle	10	341.4 ± 47.3	349.0 ± 96.5
High	10	342.2 ± 61.6	266.4 ± 86.3
$AgNO_3$	9	298.9 ± 64.6	249.1 ± 72.7

The results were expressed as the mean ± SD. For pups values are the average of three pups in each litter. Blood samples were pooled from all pups in each litter.

Table 4.11. CAT activities in liver of dams and pups.

Groups	No. of dams (litters)	CAT (kU/g protein)	
		Dams	Pups
Control	9	340.9 ± 148.2	205.7 ± 30.1
Low	8	247.5 ± 116.7	232.9 ± 29.8
Middle	10	220.3 ± 70.4	219.7 ± 48.1
High	10	200.1 ± 58.9	195.6 ± 40.9
$AgNO_3$	9	291.5 ± 72.1	177.1 ± 46.6

The results were expressed as the mean ± SD. For pups values are the average of three pups in each litter. n is composed from the average of three pups in each litter for pups.

Table 4.12. CAT activities in Brain of dams and pups.

Groups	No. of dams (litters)	CAT (kU/g protein)	
		Dams	Pups
Control	9	4.6 ± 1.5	13.2 ± 3.4
Low	8	4.9 ± 2.1	12.7 ± 0.9
Middle	10	5.2 ± 1.8	12.7 ± 2.8
High	10	4.7 ± 2.3	12.0 ± 2.1
$AgNO_3$	9	4.6 ± 1.6	10.6 ± 1.4

The results were expressed as the mean ± SD. For pups values are the average of three pups in each litter. n is composed from the average of three pups in each litter for pups.

GPx

GPx activities of erythrocyte, liver and brain homogenates of dams and pups were depicted in Figure 4.10 and 4.11 respectively. Significant decrease in GPx level was observed in $AgNO_3$ exposed dams comparing to all Ag-NPs treated groups and control ($p < 0.001$) in the liver. Also GPx level was significantly ($p < 0.05$) increased in $AgNO_3$ group versus to control in the brain. GPx levels were found similar between groups in the erythrocyte ($p > 0.05$). Also no statistically significant change was found between Ag-NPs treated dams and control in either liver or brain homogenates ($p > 0.05$).

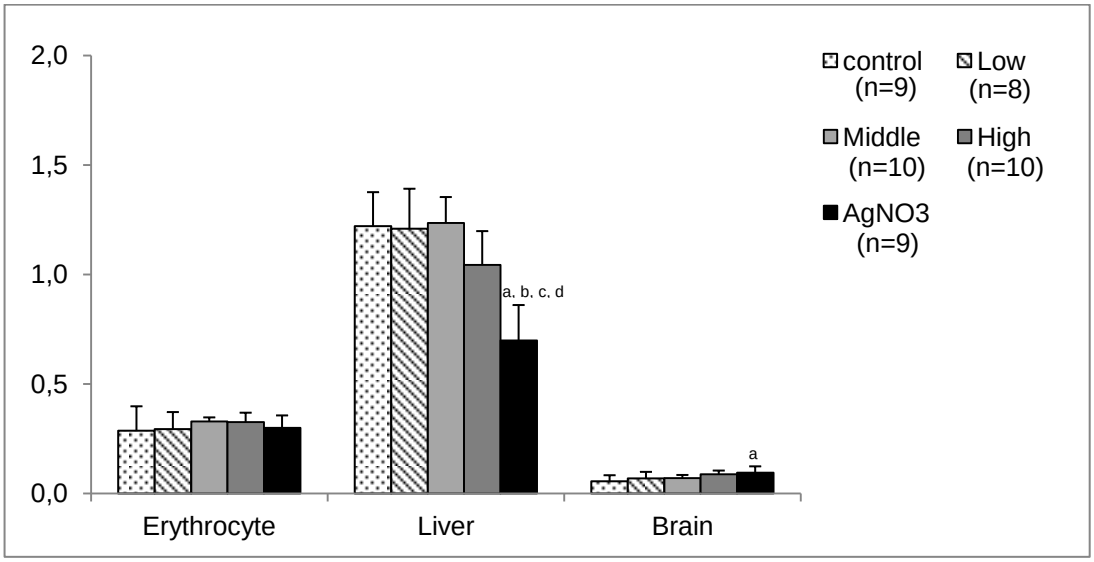


Figure 4.10. GPx activities of erythrocyte, liver and brain homogenates of dams (kU/g protein). Results were expressed as the mean ± SD. a: significantly different from control ($p < 0.05$), b: significantly different from low dose ($p < 0.05$), c: significantly different from middle dose ($p < 0.05$), d: significantly different from high dose ($p < 0.05$).

As shown in Figure 4.10, GPx levels were also evaluated in pups (Figure 4.11). $AgNO_3$ group revealed significant difference ($p < 0.01$) to all Ag-NPs groups in the erythrocyte. However, this difference was not significant versus to control ($p > 0.05$). Also GPx activity was decreased in $AgNO_3$ group comparing to all other groups in liver homogenate ($p < 0.05$). In the brain homogenate, GPx levels were increased significantly ($p < 0.01$) in $AgNO_3$ treated dams comparing to control.

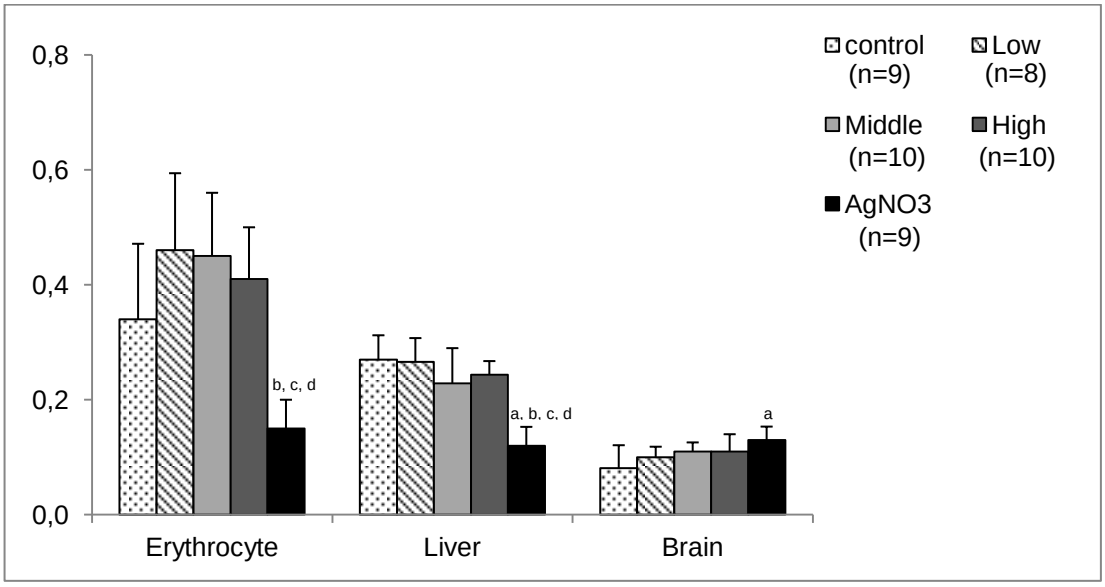


Figure 4.11. GPx activities of erythrocyte, liver and brain homogenates of pups (kU/g protein). n is composed from the average of three pups in each litter for pups' liver and brain. Blood samples were pooled from all pups in each litter. The results were expressed as the mean of three pups in each litter ± SD. a: significantly different from control ($p < 0.05$), b: significantly different from low dose ($p < 0.05$), c: significantly different from middle dose ($p < 0.05$), d: significantly different from high dose ($p < 0.05$).

4.2.8. Lipid Peroxidation Levels in Dams and Pups

MDA, as an end product and reliable marker of lipid peroxidation, was measured in erythrocyte, liver and brain homogenates. MDA levels of dams and pups were represented in Figure 4.12 and 4.13 respectively. No statistically significant differences were found between groups in erythrocyte as well as liver and brain homogenates either in dams or pups.

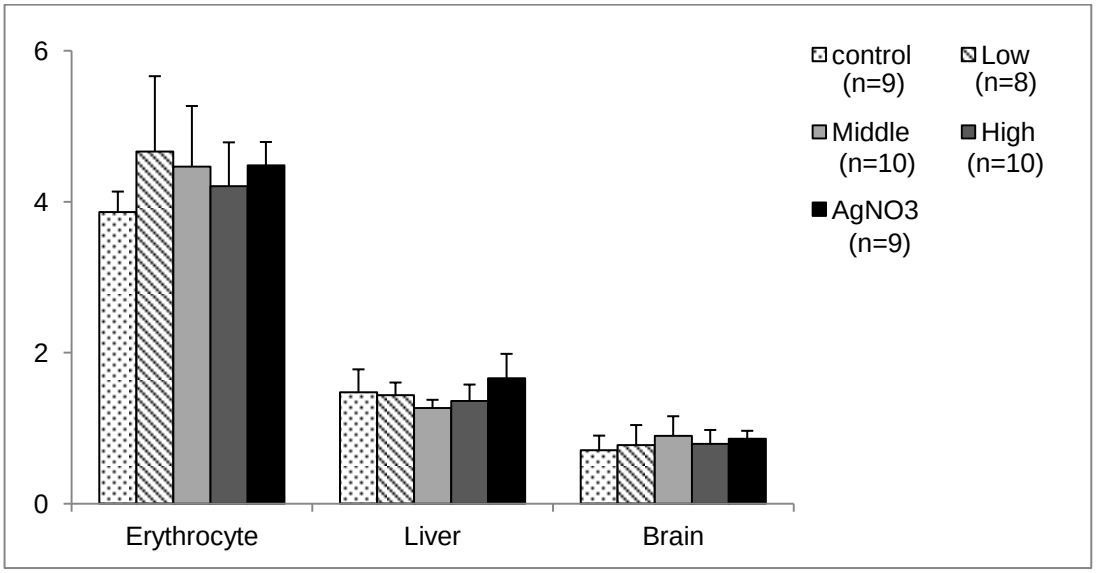


Figure 4.12. MDA levels of erythrocyte, liver and brain homogenates in dams (nmol/g protein). The results were expressed as the mean ± SD.

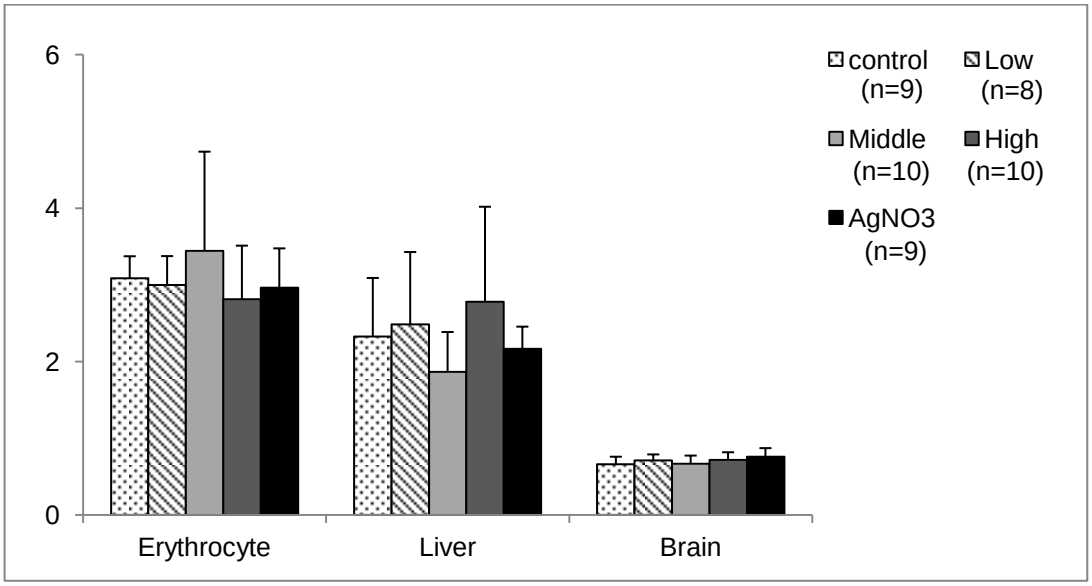


Figure 4.13. MDA levels of erythrocyte, liver and brain homogenates in pups (nmol/g protein). n is composed from the average of three pups in each litter for pups' liver and brain. Blood samples were pooled from all pups in each litter. The results were expressed as the mean of three pups in each litter ± SD.

4.2.9. NO_2^-/NO_3^- and 3-NT Levels in Dams and Pups

NO_2^-/NO_3^- concentrations in plasma samples of both dams and pups were given in Figure 4.14. Results revealed a significant increase ($p < 0.01$) in NO_2^-/NO_3^- levels of $AgNO_3$ exposed dams comparing to all NPs treated and control groups. No significant differences were observed in pups.

3-NT concentration in plasma samples of dams and pups was summarized in Table 4.13. No statistically significant change was observed in either dams or pups.

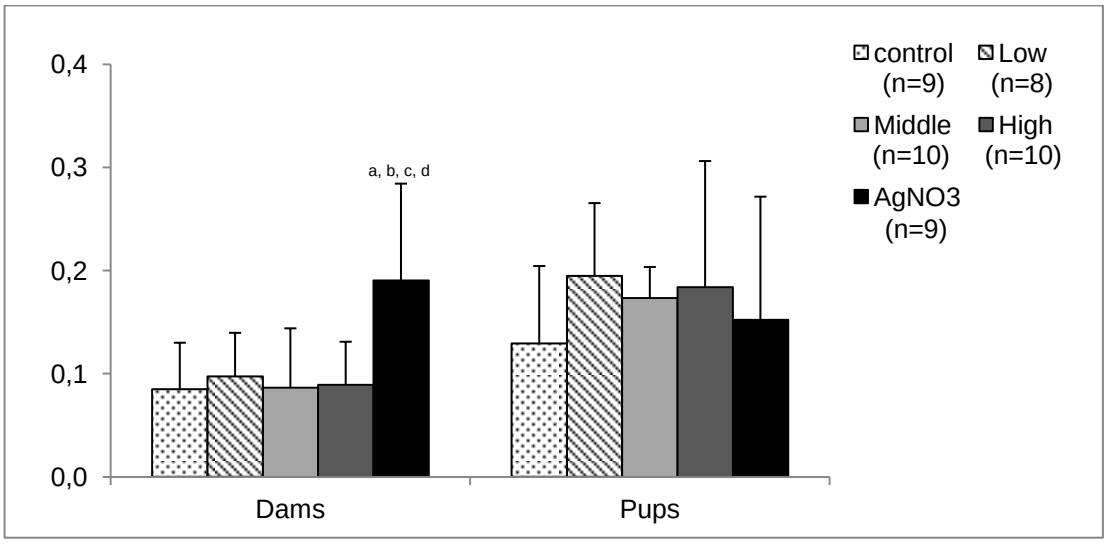


Figure 4.14. NO_2^-/NO_3^- levels in dams and pups (nmol/mL). The results were expressed as the mean ± SD. Blood samples were pooled from all pups in each litter. a: significantly different from control ($p < 0.05$), b: significantly different from low dose ($p < 0.05$), c: significantly different from middle dose ($p < 0.05$), d: significantly different from high dose ($p < 0.05$).

Table 4.13. 3-NT levels in dams and pups.

Groups	3-NT (nmol/L)	
	Dams	Pups
Control (n=9)	236.7 ± 86.8	139.6 ± 59.0
Low (n=8)	246.4 ± 101.0	92.6 ± 38.4
Middle (n=10)	278.1 ± 18.6	135.6 ± 30.3
High (n=10)	292.6 ± 75.5	121.1 ± 73.5
$AgNO_3$ (n=9)	253.2 ± 59.7	183.0 ± 71.8

The results were expressed as the mean ± SD.

4.2.10. MT-2 Levels in Dams and Pups

MT-2 concentration in plasma samples of dams and pups was illustrated in Figure 4.15. No statistically significant differences were found between groups in either dams or pups.

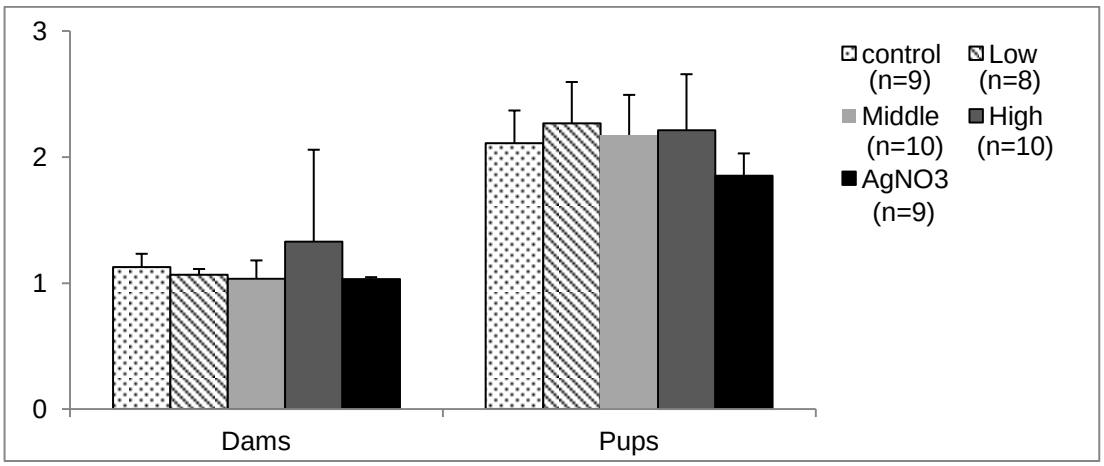


Figure 4.15. MT-2 levels in dams and pups (ng/mL). The results were expressed as the mean ± SD. Blood samples were pooled from all pups in each litter.

4.2.11. Progesterone, Estradiol and Oxytocin Levels in Dams

Results of progesterone, estradiol and oxytocin levels in plasma of the studied dams were presented in Table 4.14. No statistically significant

change was observed between groups in progesterone, estradiol and oxytocin levels.

Table. 4.14. Progesterone, estradiol and oxytocin levels in dams.

Groups	Progesterone (ng/mL)	Estradiol (ng/L)	Oxytocin (pg/mL)
Control (n=9)	9.8 ± 4.1	137.4 ± 24.7	292.1 ± 30.0
Low (n=8)	10.6 ± 4.8	142.9 ± 18.5	289.6 ± 19.7
Middle (n=10)	11.9 ± 3.3	154.4 ± 22.7	289.3 ± 12.7
High (n=10)	10.0 ± 2.4	151.3 ± 16.1	293.9 ± 11.8
$AgNO_3$ (n=9)	10.4 ± 4.1	145.5 ± 12.8	300.1 ± 19.4

The results were expressed as the mean ± SD.

4.2.12. Genotoxicity in Dams

Single strand DNA break was studied by using alkaline comet assay. The tail intensity was found similar ($p > 0.05$) in all Ag treated groups compared to control (Table 4.15, Figure 4.16).

Table 4.15. Tail intensity resulted from comet assay.

	Dose levels					
	Control	0.2 mg/kg Ag-NPs	2 mg/kg Ag-NPs	20 mg/kg Ag-NPs	20 mg/kg $AgNO_3$	Positive control
n	5	5	5	5	5	5
Tail intensity	0.40 ± 0.17	0.34 ± 0.09	0.41 ± 0.13	0.31 ± 0.09	0.32 ± 0.12	58.66 ± 17.66*

The results were expressed as the mean ± SD. * $p < 0.05$ comparing to all groups.

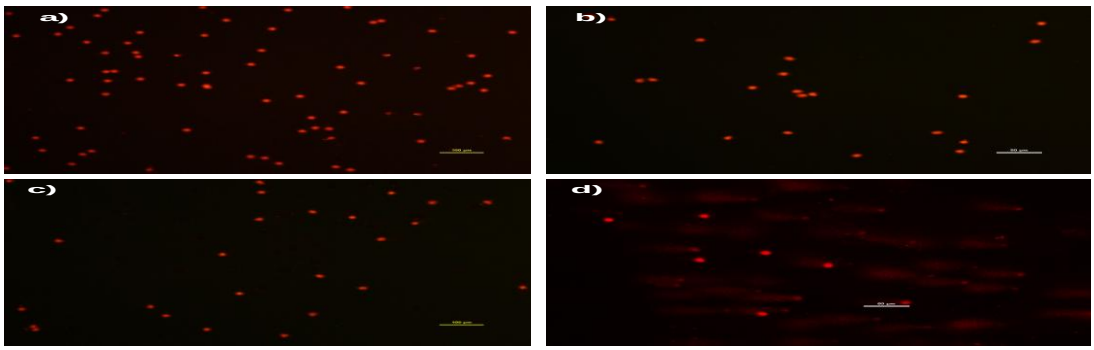


Figure 4.16. Comet assay from blood samples of control (a), 20 mg/kg Ag-NPs treated (b), 20 mg/kg $AgNO_3$ treated (c) dams and positive control (d).

4.2.13. Histopathologic Examination

Histopathological findings in liver, kidney, lung, spleen, heart and uterus tissues for dams were summarized in Table 4.16.

In dams' liver, minimal hepatocellular vacuolation was identified in 0/5, 2/5, 1/5, 2/5 and 3/5 of control, low, middle, high dose treated Ag-NPs and $AgNO_3$ group, respectively. No incidence of bile-duct hyperplasia, necrosis and inflammation or other abnormality was seen in dams' liver.

In dams' kidney, minimal interstitial inflammation was observed in 0/5, 1/5, 0/5, 2/5 and 2/5 of control, low, middle, high dose treated Ag-NPs and $AgNO_3$ group, respectively. Also minimal tubular necrosis was found in 0/5, 1/5, 4/5, 3/5 and 3/5 of control, low, middle, high dose treated Ag-NPs and $AgNO_3$ group, respectively (Figure 4.17).

Histopathological examination of dams' lung tissue revealed minimal and mild inflammation in 0/5, 1/5, 0/5, 2/5 and 2/5 of control, low, middle, high dose treated Ag-NPs and $AgNO_3$ group, respectively (Figure 4.18). Also 0/5, 2/5, 1/5 and 3/5 of control, low, middle and high dose exposed Ag-NPs dams showed histiocytosis accumulation as well as anthracosis (Figure 4.19). 1/5 and 2/5 of $AgNO_3$ treated group was shown histiocytosis and anthracosis, respectively.

In dams' spleen minimal periarterial hyalinization was observed in 0/5, 0/5, 1/5, 2/5 and 2/5 of control, low, middle, high dose treated Ag-NPs and $AgNO_3$ group, respectively.

Histopathological examination of heart and uterus tissues did not show any treatment related effects.

Table 4.16. Histopathological findings for dams.

Group				Control	Low	Middle	High	$AgNo_3$
Number of animals (n)				5	5	5	5	5
				n	n	n	n	n
Liver	No microscopic findings			5/5	3/5	4/5	3/5	2/5
	Abnormality							
	Hyperplasia	Bile duct		0/5	0/5	0/5	0/5	0/5
	Vacuolation	hepatocellular	minimum	0/5	2/5	1/5	2/5	3/5
	Necrosis			0/5	0/5	0/5	0/5	0/5
	Hemorrhage			0/5	0/5	0/5	0/5	0/5
	Pigmentation			0/5	0/5	0/5	0/5	0/5
	Inflammation			0/5	0/5	0/5	0/5	0/5
Kidney	No microscopic findings			5/5	4/5	1/5	1/5	1/5
	Abnormality							
	Basophilia			0/5	0/5	0/5	0/5	0/5
	Inflammation		minimum	0/5	1/5	0/5	2/5	2/5
	Necrosis	tubular	minimum	0/5	1/5	4/5	3/5	3/5
Lung	No microscopic findings			5/5	2/5	3/5	1/5	1/5
	Abnormality							
	Inflammation		minimum	0/5	0/5	0/5	1/5	2/5
			mild	0/5	1/5	0/5	0/5	1/5
	Histiocytosis accumulation		minimum	0/5	2/5	1/5	3/5	1/5
	Mineralization			0/5	0/5	0/5	0/5	0/5
	Anthracosis			0/5	2/5	1/5	3/5	2/5
Spleen	No microscopic findings			5/5	5/5	4/5	3/5	2/5
	Abnormality							
	Infarct			0/5	0/5	0/5	0/5	0/5
	Inflammation			0/5	0/5	0/5	0/5	0/5
	Hyalinization	periarterial	minimum	0/5	0/5	1/5	2/5	3/5
Heart	No microscopic findings			5/5	5/5	5/5	5/5	5/5
Uterus	No microscopic findings			5/5	5/5	5/5	5/5	5/5

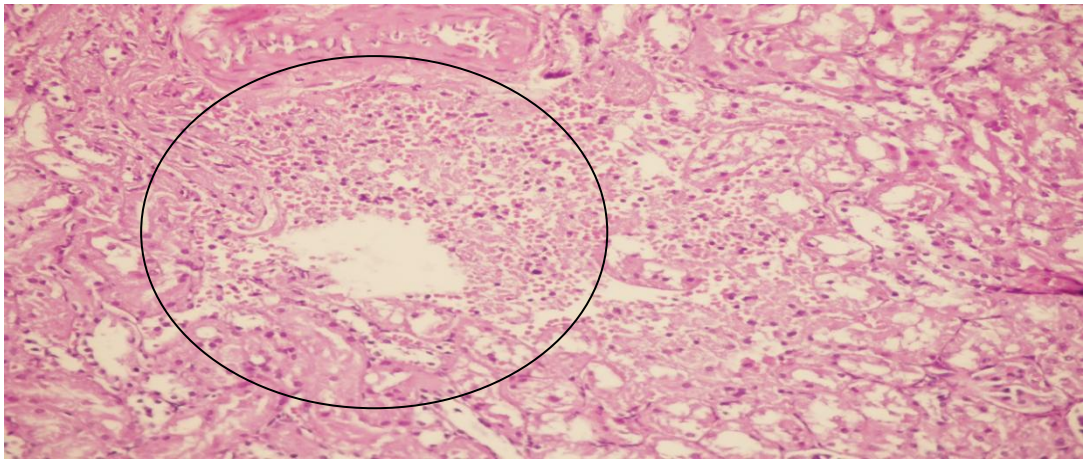


Figure 4.17. Tubular necrosis in 20 mg/kg Ag-NPs treated dam (x100).

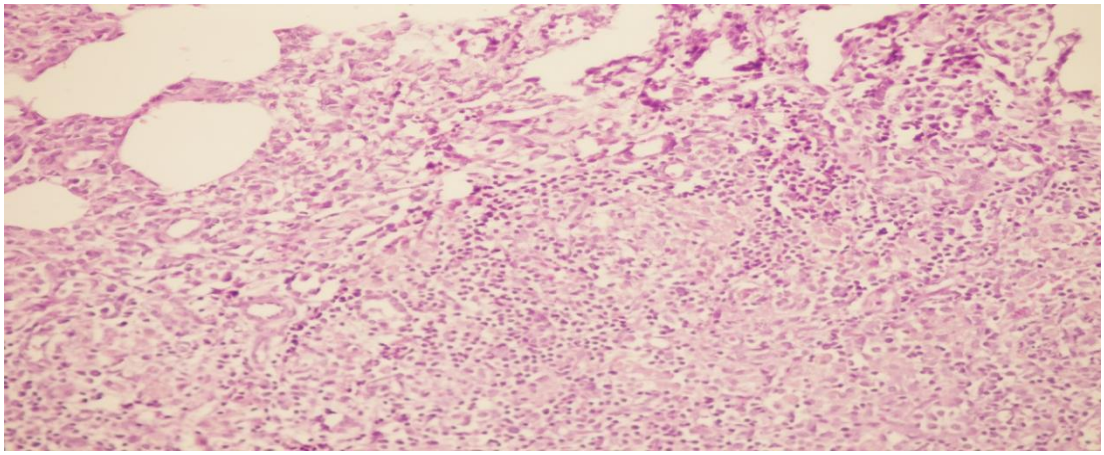


Figure 4.18. Mononuclear inflammatory cell infiltration in lung of 20 mg/kg Ag-NPs treated dam (x200).

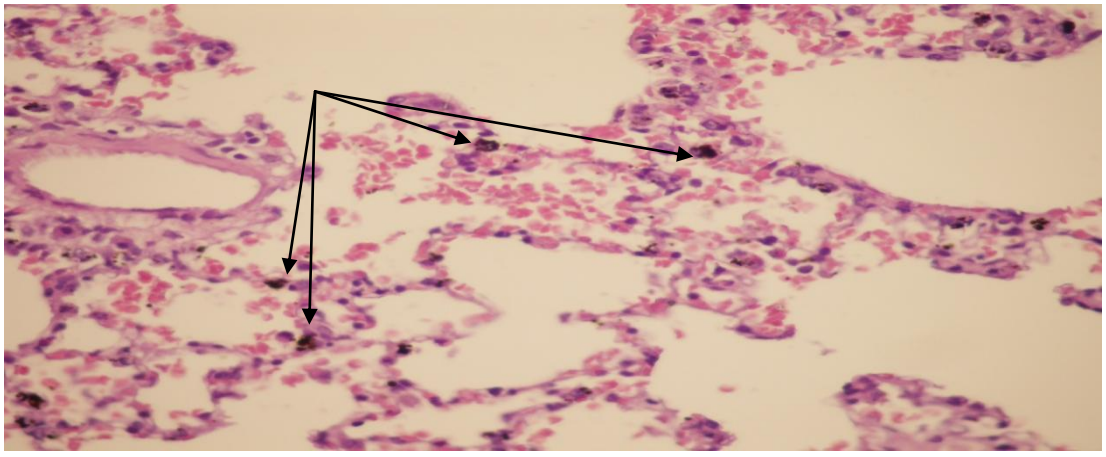
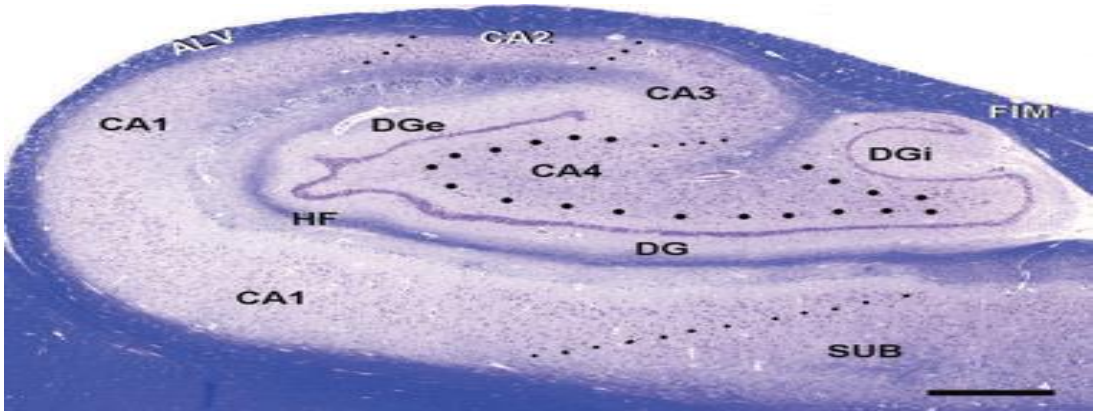


Figure 4.19. Pigment accumulation (anthracosis) in lung of 20 mg/kg Ag-NPs treated dam (x200).

Histopathological findings in brain tissue of dams revealed the high incidence of hippocampal pyramidal cell loss (hippocampal sclerosis) in both Ag-NPs and $AgNO_3$ treated groups.

Hippocampal sclerosis (HS) findings, visualized by histopathologic examination of dams' brain, were classified according to the International League against Epilepsy (ILAE) (Figure 4.20) and represented in Table 4.17 and Figure 4.21 (196, 197).



Sectors	Neuronal cell loss / Gliosis pattern			
DG	0-2	1-2	0-2	0-2
CA4	2	2	0-1	1-2
CA3	0-1	2	0-1	0-1
CA2	0-1	1-2	0-1	0-1
CA1	2	2	0-1	0-1
SUB	0-1	0-1	0	0
ILAE classification	Type 1a (classic HS)	Type 1b (severe HS)	Type 2 (CA1 HS)	Type 3 (CA4 HS)

Figure 4.20. Microscopic anatomy of the human hippocampus and ILAE consensus classification of hippocampal sclerosis. SUB, subiculum; CA1 CA4, sectors of the Cornu ammonis; DG, dentate gyrus with external (DGe) and internal limbs (DGi); HF, remnant of hippocampal fissure; ALV, alveus; FIM, fimbria. Dotted lines circumscribe anatomic boundaries between CA sectors, and cell quantification. The scoring system refers to neuronal cell loss and is defined for CA1–CA4 as following: 0 = no obvious neuronal loss or moderate astrogliosis only; 1 = moderate neuronal loss and gliosis (GFAP); 2 = severe neuronal loss (majority of neurons lost) and fibrillary astrogliosis (196, 197).

Table 4.17. Histopathological findings for dams' Brain.

Groups	Control	Low	Middle	High	$AgNo_3$
Number of animals (n)	5	5	5	5	5
	n	n	n	n	n
No microscopic findings	5/5	1/5	2/5	1/5	1/5
Number of dams with neuronal loss event	0/5	4/5	3/5	4/5	4/5
ILAE classification					
Type 1a	0/5	1/4	2/3	1/5	2/4
Type 1b	0/5	2/4	0/3	1/5	1/4
Type 2	0/5	1/4	1/3	2/5	1/4

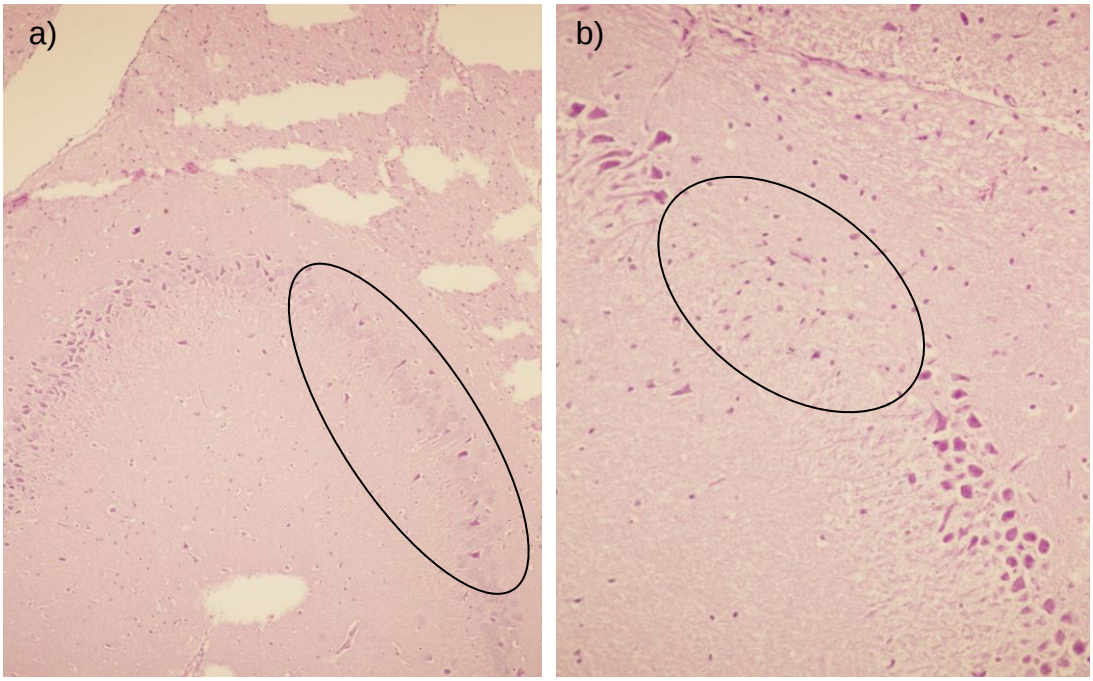


Figure 4.21. Hippocampus of 20 mg/kg Ag-NPs treated dams with loss of neurons in CA sector. a) X100, b) X200.

Histopathological examination of brain, heart, liver, kidney and lung tissues of offspring did not show any treatment related effects. Just in one pup from 2 mg/kg Ag-NPs group, minimal anthracosis was observed in lung tissue.

5. DISCUSSION

Engineered nanomaterials offer technological advantages for a variety of consumer products and commercial processes. Since Ag is an element with no recognized function in the human body, exposure to it is considered to be unwanted (198). However, due to the ideal antimicrobial and unique physical characteristics, Ag-NPs are increasingly used in a vast range of consumer products (3). The rapidly increasing applications of Ag-NPs, has recently raised concerns over the potential health impacts of increased human exposure to this material. Therefore, information regarding the safety and potential hazards of Ag-NPs is crucial, particularly for vulnerable and susceptible subpopulations such as pregnant women and their developing fetuses.

The evaluation of toxicity of Ag-NPs in some *in vitro* and *in vivo* studies has revealed the association of these NPs exposure with oxidative, inflammatory, cytotoxic, genotoxic and consequences (6). However, very little is known about the potential toxicity of Ag-NPs on dams and offspring during the prenatal exposure. Furthermore, data on whether Ag-NPs could cross the placental barrier is still limited.

In the present study, the maternal and developmental toxic potential of orally administered Ag-NPs to Sprague-Dawley rats from GD 7-20 was investigated.

Rat model is used in this study due to its genomic similarity to humans, short generation time as well as large litter size (181).

Oral route was selected due to the extensive use of Ag-NPs in food/ feed industry, water treatment (3, 199), toothpaste, reusable bottles, kitchen utensils and toys (169).

Doses of 0, 0.2, 2 and 20 mg/kg/day were selected in this study. The relevance of dose/ concentration levels is one of the critical parameters for assessing safety of NPs (25). Up to 1000 mg/kg/day dose level was utilized

in a 28-day study on oral toxicity of Ag-NPs and NOAEL value of 30 mg/kg was suggested by authors (169). Similarly, Yu et al conducted a study to investigate effects of Ag-NPs on pregnant rats as well as embryo-fetal development using up to 1000 mg/kg dose levels. Authors suggested the NOAEL values less than 100 mg/kg/day for dams and 1000 mg/kg/day for embryo-fetal development (187). Contrariwise, Johnston et al found these dose levels unrealistic to human situation and highlighted the importance of experimental design impact on the outcomes of the conducted studies (6). Although it is difficult to predict human exposure level to Ag-NPs, some reports estimate level of < 100 µg/day as a realistic dose for Ag-NPs toxicity studies (200).

Present study, was attempted to identify the potential adverse effects of Ag-NPs in exposure levels below the NOAEL value which suggested in previous studies. Although concentrations utilized within present study were above the estimated suggested realistic levels, these utilized doses are not exaggerated considering the short period of exposure (14 days). Furthermore, additional group of dams were treated with $AgNO_3$ at the same concentration with high dose Ag-NPs (20 mg/kg) group in order to compare possible toxicity of Ag-NPs with Ag ions.

It may be argued that, the amount of Ag in ionic form present in the prepared NP suspension should be also measured, as the influence of Ag ions on the results of this study must not be underestimated. In order to eliminate the possibility of Ag ions presence in the prepared suspensions, approximately 3 times the required trisodium citrate was utilized during the syntheses process of Ag-NPs. According to the chemical reaction of synthesis process, in order to convert the total amount of Ag (57mg) that was used in this research to prepare Ag-NPs, 34 mg of trisodium citrate was required nevertheless we utilized 100 mg of trisodium citrate in order to ensure all the Ag ions were converted to the particulate form of Ag.

Following the synthesis process, the NPs were rinsed three times to ensure any remaining Ag ions were dissolved in the liquid form and removed

from the samples. To ensure the original Ag content of the sample was kept in the suspension during the rinsing process, the Ag content of the prepared suspension was measured by atomic absorption spectrometer method. Results indicated that 104% of the original Ag content was present in the sample. In a different approach, specific volume of suspension was imported into a falcon tube which was then reserved in an oven at 30°C for 24 hours until all the liquid was evaporated. The remaining content was weighed and it was found that 98% of the Ag content was present in the dried content.

5.1. Findings on Pregnancy and Fetuses

A number of previous studies have demonstrated that Ag-NPs create abnormalities on embryonic development in zebrafish (182) and lead to enhancement in resorption rate of post-implantation embryos as well as a reduction in fetal weight in mice (184). Philbrook et al reported that Ag-NPs decreased viability of fetal mice when given orally at 10 mg/kg to pregnant mice, but not at higher dose levels (183). In the present study, no treatment related effects were found for the reproductive parameters including pregnancy length, maternal weight gain, and number of implants, fetal weight and litter size (Table 4.1). These findings support previous reports that demonstrates no signs of reproductive/ developmental toxicity up to 1000 mg/kg/day (186, 187).

5.2. Organ Weights

No significant relative and absolute organ weight changes were observed in either dams or offspring except for an increase ($p < 0.05$) in the relative weight of the brain, heart and uterus for the high dose Ag-NPs treated and in the absolute weight of uterus ($p < 0.05$) for the middle dose Ag-NPs treated dams (Table 4.2-4.5). Slight increase in absolute brain weights of orally Ag-NPs treated dams (100, 300 and 1000 mg/kg/day) were also found in a rat embryo-fetal developmental study conducted by Yu et al (187). Authors considered that this increase is incidental and not treatment-related, as the changes did not show a dose-response relationship. However,

similar finding in relative brain weight in present study indicate that this may be an Ag treatment related effect.

5.3. Ag Content in Organs

Previously published works have reported that Ag-NPs can be absorbed into blood circulation and accumulated in many tissues, including liver, kidneys, lungs, spleen, brain, blood, ovaries and testis following oral (166-168, 201), inhalation (173) and intravenous (177) exposure. However, the distribution of Ag-NPs into maternal tissues during pregnancy as well as possible transfer of these NPs to fetus through the placenta barrier is still curious issue.

In the present study, the maternal blood Ag content in the Ag-NPs treated groups increased significantly in a dose dependent manner. The high Ag concentration in plasma comparing to the erythrocyte corroborates a previous report indicating a high degree of serum proteins binding for Ag (202). On the other hand, the maternal blood Ag level in the $AgNO_3$ group was significantly higher than that of the Ag-NPs group with the similar Ag level (Table 4.6 and Figure 4.5). This could illustrate a higher gastrointestinal absorption rate of Ag in the form of $AgNO_3$ when compared to particulate form. This corroborates with Loeschner et al findings in a 28-day study in which rats were orally exposed to PVP-coated Ag-NPs and Ag acetate. In that study authors found lower Ag levels in tissues following oral administration of Ag-NPs compared to Ag acetate (165). This finding might be explained by the enhanced binding of NPs to non-digestible food components in the intestines because of larger active surface, and therefore the smaller absorption.

It is likely that, the Ag-NPs dissociate to a higher extent in vivo, compared to in vitro, during the digestive process due to the physiological parameters such as pH. However, in a recent study using an artificial digestion model, it was shown that Ag-NPs survive the gastrointestinal juices in their particulate form without forming large quantities of aggregates (200).

Also, van der Zande et al reported that the number, size and the composition of Ag-NPs were not affected by differences in pH (166).

The much lower Ag content of maternal blood than tissue levels indicated that the Ag was cleared from the blood and distributed to the other organs (Table 4.6 and Figure 4.5). In Ag-NPs treated groups, maternal spleen and kidney were the organs with the highest Ag accumulation with 9.3 and 5.4 fold increases in high dose group followed by liver, uterus and heart (approximately 2.5 fold) versus controls (Table 4.6). The concentration of Ag in tissues corroborates with the blood Ag level which was much higher in the $AgNO_3$ treated group than Ag-NPs group. Some previous studies demonstrated that liver and kidney are the main target organs for silver distribution upon oral exposure of Ag-NPs (165-168). Similarly, Austin et al reported that maternal liver and spleen are the major sites of Ag-NPs accumulation following intravenous injection to pregnant mice (203). Considering the observed particle size of Ag-NPs suspension (70 nm) in current study, the high accumulation of Ag in maternal spleen can be also interpreted as a size dependent distribution of Ag-NPs to the spleen, since a previous study revealed that 80 and 110 nm particles resulted in high distribution in spleen, while 20 nm particles did not (177). The high concentration of Ag in maternal kidney also is in corroboration with previous studies (167, 168, 201). For example, the results of Kim et al demonstrated 5.6 fold higher concentration of Ag in female kidneys compared to that of liver (167). Furthermore, it should be considered that besides physicochemical characteristics of NPs utilized in different studies, also the animal species, strain, age, breeding, housing and inter-animal differences may have an impact on different distribution patterns (166).

Increase in renal Ag levels of dams reported in the present study may be consistent with histopathological findings of the maternal kidney tissue which revealed a relatively high incidence of tubular necrosis (Table 4.16 and Figure 4.17). Furthermore, the ingestion of Ag-NPs has been demonstrated

to cause preferential accumulation of Ag at high levels in the basement membranes of the renal tubules in female rats (201).

As shown in Figure 4.6 and Table 4.7, accumulation of Ag was observed in the tissues of offspring from the treated dams indicating the ability of Ag to cross the placental barrier. The kidney seemed to be the main fetal accumulation target and exhibited 5.8 fold higher Ag concentrations compared to control in the pups from high dose Ag-NPs treated dams. Besides high dose Ag-NPs group, statistically significant ($p < 0.01$) increase in the kidney Ag content of middle and even low dose Ag-NPs groups was also observed. The Ag level of kidney in high dose Ag-NPs group was followed by the lung (4.3 fold versus to control), liver (3.2 fold versus to control) and brain (2.0 fold versus to control).

The organ distribution pattern and levels of Ag in pups were comparable to the results from a short communication conducted by Lee et al where 250 mg/kg/day citrate-coated Ag-NPs of 7.9 nm in diameter were administered orally to pregnant rats for 14 days before mating, during the mating period and gestation period, and finally 4 days after parturition (204). The fact that Ag increase in exposed groups versus controls in the Lee et al study were just approximately 2 times higher than in our study was interesting because they used a higher dose and smaller particle size. This observation as well as similar Ag contents for all Ag-NPs exposure levels (Figure 4.6 and Table 4.7) might be pinning the influence of dose and particle size on the Ag-NPs transfer to fetus through the placenta barrier.

Furthermore, in line with our findings, Lee et al revealed that the offspring kidney is the main target for Ag accumulation following oral administration of Ag-NPs to dams during pregnancy (204).

In contrast to distribution pattern findings in dams, the similar or even lower organ content of Ag in offspring of $AgNO_3$ group compared to Ag-NPs groups was surprising (Figure 4.6 and Table 4.7).

This observation may be explained by the size dependent translocation of Ag in nano-particulate and ionic forms across the placental barrier. Also the lack of dose dependency in Ag content of offspring tissues may be related to a maximum transfer limit of Ag through placenta. However, blood Ag levels still remained elevated in $AgNO_3$ group in comparison with that of Ag-NPs group (Table and Figure 4.7).

This controversy might be explained by Ag concentrations in plasma and erythrocyte of offspring in high dose Ag-NPs and $AgNO_3$ groups. As given in Table and Figure 4.7, the same Ag content was detected in the erythrocyte and plasma of $AgNO_3$ groups, whereas the Ag content of plasma in Ag-NPs group was reduced by half in that of erythrocyte. Assuming that ionic Ag was bonded to plasma proteins and was distributed to other tissues with more difficulty, Ag in NP form could maybe distribute and be taken up more easily than Ag^+.

To our knowledge, this is the only study comparing the distribution pattern of Ag in rat offspring organs following oral administration of Ag-NPs or a soluble Ag salt in pregnant rats. According to our results, it is unlikely that the Ag-NPs all immediately dissolved into silver ions, as this would have resulted in a similar distribution pattern. This finding illustrates that a fraction of administered dose may have reached to pups in nano-particulate form. However, further studies are needed to gather information on the detailed mechanisms of the Ag-NPs transport system.

Furthermore, as shown in Table 4.8, Ag concentrations in stomach milk of offspring demonstrated no statistically significant ($p > 0.05$) differences between Ag-NPs as well as $AgNO_3$ treated groups versus controls. However, Ag increase in milk samples was observed in both Ag-NPs and $AgNO_3$ groups, suggesting the possible transfer of Ag from dams to the offspring through lactation period.

5.4. Biomarkers of Hepatotoxicity

The results of the biochemical analysis of two biomarkers of hepatotoxicity, ALT and AST, in plasma showed no significant differences between groups, which may be determinative of no acute hepatotoxicity indication. However, in spite of not significant increment in ALT and AST levels, some minimal adverse responses were still observed in histopathological analysis of liver (Table 4.9). Our findings on the ALT and AST levels are compatible with the report by van der Zande et al where no changes in ALT and AST levels were found after 28-day oral exposure to Ag-NPs (90 mg/kg/day) and $AgNO_3$ (9 mg/kg/day) in rats (166). No histopathological examination was conducted in that study.

5.5. Oxidative Stress

For the purpose of evaluating the levels of oxidative stress induced by Ag-NPs in dams and offspring, we measured the activities of enzymes SOD, CAT and GPx. These enzymes form the first line of defense against ROS by converting the reactive oxygen species into non-toxic compounds (133). SOD catalyzes the conversion of $O_2^{-\bullet}$ to H_2O_2 and molecular oxygen. CAT further detoxifies H_2O_2 to water. GPx also catalyzes the reduction of H_2O_2 into water using GSH as the reducing substrate (136, 205, 206). Hence, these enzymes compose the enzymatic antioxidant capacity against ROS.

During oxidative stress, membrane lipids are continuously subjected to oxidative stress. Hence, the measurement of lipid peroxidation is an important indicator in the assessment of antioxidant potential. We evaluated the amount MDA, which is one of the most important by-products of lipid peroxidation (133).

A growing amount of data shows the role of oxidative stress in mediation of cytotoxicity, DNA damage and apoptosis induced by Ag-NPs (6, 147, 149, 179, 207, 208). However, the knowledge about NO radical related parameters which contribute to the toxicity of Ag-NPs has not been investigated previously.

NO is a nonstable radical and converted to NO_2^-/NO_3^- which is more stable product. However, excess NO reacts with $O_2^{-\bullet}$ to give $ONOO^-$ which leads to nitration of aromatic amino acid residues, thereby exerts cytotoxic and cytostatic effects *in vivo*. 3-NT is one of the important biomarkers of $ONOO^-$ mediated (NO - dependent) damage (189, 209). Hence, in this study, NO_2^-/NO_3^- as a stable product of NO radical as well as 3-NT were assessed in plasma samples of both dams and offspring to evaluate the NO-related parameters possibly elevated by Ag-NPs exposure.

Administration of $AgNO_3$ to pregnant rats resulted in significant decrease ($p < 0.05$) in SOD activity of liver comparing to control and low dose Ag-NPs treated dams (Figure 4.8). Compare to the control group, this decrease in liver SOD (~ 25%) was very close to that of the decrease in erythrocytes (~ 20%). However, this difference was not found statistically significant in erythrocyte. This inhibition in SOD activity could be due to the flux of $O_2^{-\bullet}$ resulting in H_2O_2 production in liver cells (206). Although no statistically significant ($p > 0.05$) changes were found either in erythrocyte or liver homogenate of Ag-NPs exposed dams, slight decrease in SOD in erythrocyte and liver homogenate was observed for NPs in dose dependent manner.

In terms of brain SOD, $AgNO_3$ group showed significant increase ($p < 0.05$) in SOD levels compared to all other groups. The reverse trend of SOD in liver and brain may be explained by Upreti et al study (2002). They reported that during the pregnancy period, the brain tissue is more susceptible to oxidative stress than other tissues due to the reduction in activity of SOD, CAT and GPx (210). Considering the failure of the total antioxidant defense mechanism, the elevated activity of brain SOD in current study may be due to the adaptive response to the generation of free radicals during Ag exposure.

In $AgNO_3$ treated dams, GPx activity of brain was increased slightly but significantly ($p < 0.05$) comparing to control (Figure 4.10). This may be explained again by adaptive response of brain tissue. In Ag-NPs treated

groups, no significant ($p > 0.05$) changes were found between groups. However, slightly increase in SOD activity of brain was observed in a dose dependent manner. In term of liver GPx, significant decrease ($p < 0.001$) was observed for $AgNO_3$ compared to control as well as all Ag-NPs treated groups. The decreased liver activity of GPx may be resulted from the involvement of this enzyme in the scavenging of peroxides generated during Ag biotransformation (211). Also GPx activity in the liver tissue of high dose Ag-NPs treated dams was decreased slightly comparing to control group, although this difference was not statistically significant ($p > 0.05$).

After treatment of rats with Ag-NPs from GD 7 to GD 20, no significant differences were observed in MDA levels in erythrocyte as well as liver and brain homogenates, signifying no indication of lipid peroxidation. These findings indicate that Ag-NPs did not exert oxidative damage in dams at the dose levels up to 20 mg/kg/day. Also no significant differences were found in NO_2^-/NO_3^- and 3-NT levels in maternal blood compared to the control. On the other hand, when $AgNO_3$ exposed dams compared to Ag-NPs and control groups, significant ($p < 0.01$) increase in plasma NO_2^-/NO_3^- levels were observed in comparison to all other groups (Figure 4.14), though it was not accompanied by changes in 3-NT. This data provide evidence suggesting that NO may have an important role in oxidative stress associated consequences arising from exposure to ionic Ag.

Together, findings of decreased levels of SOD and GPx levels in dam liver and of increased NO_2^-/NO_3^- levels in plasma for $AgNO_3$ indicates that Ag induce oxidative stress and more so when administered in ionic than in nano-particulate form.

In term of offspring, Investigation of indicators of oxidative stress including SOD, CAT and GPx in the erythrocyte, liver and brain demonstrated no apparent increase in ROS production. Also no significant changes were found in NO_2^-/NO_3^-, 3-NT and MDA levels either in blood or tissue homogenates. These findings indicate that Ag-NPs do not cause oxidative

stress/ damage in offspring rats following gestational exposure to Ag-NPs at the dose levels utilized in this study.

However, similar findings to dams in SOD and GPx activities were observed in offspring following gestational exposure to $AgNO_3$. As shown in figure 4.9, slightly decrease in SOD activity was found in erythrocyte and liver homogenate comparing to control. Also a slight increase in brain SOD activity was observed similarly to dams. However, these differences were not statistically significant ($p > 0.05$). In term of GPx, results showed a significant decrease in erythrocyte ($p < 0.01$) and liver homogenate ($p < 0.05$) as well as increase ($p < 0.01$) in brain tissue (Figure 4.11).

In addition, administration of $AgNO_3$ to pregnant rats did not cause any changes in CAT activity either in dams or pups compared to control. CAT is extensively involved in the degradation of H_2O_2 which is resulted from dismutation of superoxides by SOD. GPx also catalyzes the reduction of H_2O_2 and lipid peroxides at the expense of GSH. Although CAT and GPx share the same substrate, GPx is more effective at low levels of H_2O_2 and CAT is more effective at high levels of H_2O_2 (212). According to our results, the decreased SOD and GPx activity and no change in CAT activity of both dams and pups' liver tissue, suggest that $AgNO_3$ in 20 mg/kg/day dose level resulted in low levels of H_2O_2 production.

Taken together, it is obvious that Ag induced oxidative stress in both dams and related offspring. However, this ability was just observed significantly when Ag was administrated to pregnant rats in ionic form since no sign of significant induced oxidative stress was found in Ag-NPs treated groups up to 20 mg/kg daily dose. This may be explained by the lower gastrointestinal uptake of Ag in nano-particulate form compared to ionic form in dams. However, similar observations were found in term of GPx activity in offspring. Considering the Ag accumulation pattern in offspring tissues, it can be concluded that induced oxidative response/ damage of Ag-NPs reported in previous studies not only dependent to NP itself, but also depend on amount of Ag ions released from NP surface.

This finding is also in accordance with some previous reports, for example, Choi et al investigated the induction of oxidative stress by Ag-NPs in the liver of zebrafish (207). In that study, zebrafish were exposed to an Ag-NPs suspension in which free Ag ions were absent at the time of treatment. Results showed that the metal-sensitive MT-2 mRNA was induced in the liver tissue of Ag-NPs treated groups. Authors suggested that this effect may be due to release of Ag ions from Ag-NPs after treatment. However, in current study no significant differences were found in blood MT-2 levels between groups even in $AgNO_3$ group (Figure 4.15). It may be due to the relatively low dose levels utilized in our study. On the other hand, in contrast to Choi et al findings, slightly decrease in blood MT-2 levels was observed in both dams and pups exposed to $AgNO_3$. This may be explained by the involvement of MT as a part of antioxidant defense system (163) against induced ROS/RNS production by Ag in $AgNO_3$ exposed group.

Toxicity of NPs is manifested by inflammation resulting from oxidative stress (6, 169). In previous study, it was reported that when mice were repeatedly exposed for 28 days by oral administration with 0.25, 0.50 and 1.00 mg/kg dose of Ag-NPs, these NPs induce cytokine (IL-1, IL-6, IL-4, IL-10, IL-12 and TGF-β) level of blood in a dose dependent manner (169). In contrast, Daniel et al reported that intra-peritoneal injection of Ag-NPs in to mice showed no immune response. However, increase in CAT activity and decrease in GSH content following administration of Ag-NPs in that study, confirming the antioxidant defense capacity of Ag-NPs (213). In current study, no statistically significant ($p > 0.05$) changes were found in IL-6 levels of plasma between groups either in dams or pups up to daily dose of 20 mg/kg. However, IL-6 was slightly increased in both Ag-NPs and $AgNO_3$ treated dams as well as related offspring versus to control (Table 4.9). $AgNO_3$ group also showed the most induction of IL-6 among Ag treated groups. This induction may be in accordance with our findings on oxidative stress parameters. Subsequently, this induction may also be in accordance with findings of histopathological examinations which demonstrated higher incidence of inflammation in the form of lymphocyte influx in both Ag-NPs and

$AgNO_3$ treated dams comparing to control (Table 4.16 and Figure 4.18). No sign of treatment related effects were observed in histopathologic examination of offspring tissue.

5.6. Hormonal Levels

In a study conducted by Li et al, pregnant rats were exposed to the NP-rich diesel exhaust. Results showed a significant decrease in the serum concentration of maternal progesterone, while that of estradiol was found higher than control group (214). In our study, no statistically significant ($p > 0.05$) differences were found in either progesterone or estradiol concentration of maternal plasma in Ag exposed dams comparing to control (Table 4.14). Accordingly, no treatment related effects were found for any reproductive/ developmental parameter examined. Slightly increase in estradiol levels was observed in Ag-NPs treated dams. However, this increase was not correlated with Ag concentration in blood samples ($p > 0.05$). More studies are still required to illuminate the possible effects of Ag-NPs in endocrine system.

5.7. Genotoxicity

There are some conflicting reports in literature regarding the *in vivo* genotoxic effects of Ag-NPs. For example, Tiwari et al reported that intravenous injection of Ag-NPs resulted in increased single and double DNA breakage in rats (178). On the other hand, Kim et al demonstrated that Ag-NPs did not induce genetic toxicity in rat bone marrow following 28-day orally administration up to 1000 mg/kg daily dose (167). In accordance to that study, single cell gel electrophoresis from blood samples did not show comet formation in both Ag-NPs and $AgNO_3$ treated dams in our study (Table 4.15 and Figure 4.16). This finding indicates that orally administered Ag do not cause genotoxicity either in nano-particulate or ionic form up to 20 mg/kg daily dose level in rat during the gestation period.

5.8. Histopathology

As shown in Table 4.17, histopathologic examination of brain tissue revealed the high incidence of hippocampal sclerosis in both Ag-NPs and $AgNO_3$ treated dams. This event was observed in 4/5, 3/5, 4/5 and 4/5 of low, middle and high dose treated Ag-NPs as well as $AgNO_3$ group, respectively. According to ILAE classification, 25% of them were categorized as a severe hippocampal sclerosis. Normal morphology of pyramidal neurons in all regions of hippocampus was observed in dams of control group.

According to the study conducted by Tang et al, Ag-NPs were induced BBB destruction and astrocyte swelling and caused neuronal degeneration after subcutaneous exposure. In addition, the presence of Ag-NPs in neuronal cells was confirmed by TEM-energy dispersive X-ray spectrometry in that study (215). In another study, Daniel et al reported that Ag-NPs cross the BBB without producing apparent toxicity but up-regulating the brain function by increasing the glutamine synthase activity of the brain which is important for neurotransmission and other activities (213).

In present study, an increase in Ag levels of brain was also observed in dose dependent manner in dams. This increment besides high incidence of hippocampal neuronal cell loss is alarming. This event is even more disturbing in light of previous studies which were reported long retention of Ag in brain tissue (166, 216).

The hippocampus plays an important role in formation of memory as well as detection of novel events, places and stimuli (217). Liu et al investigated neurobehavioral effects of Ag-NPs following 14-day nasal administration. Results demonstrated that Ag-NPs induced learning and memory deficits in both low (3 mg/kg) and high (30 mg/kg) dose groups. Also histopathological observations showed a clear edema and nuclear shrink phenomenon as well as neorobiosis among the pyramidal neurons in regions of hippocampus. This was further supported by induction of superoxide anion and hydroxyl radical in hippocampus. Authors suggested that oxidative stress

may be the reason of the neuronal damage caused by Ag-NPs (218). However, it is not clear that these effects are due to the NPs or Ag ions since soluble Ag control did not included in that study.

In present study, we confirm that orally administration of both nano-particulate and aqueous forms of Ag can generate hippocampal neuronal damage. Moreover, although only administration of Ag in soluble form resulted in induced oxidative stress, similar incidence of hippocampal sclerosis was observed in nano-particulate treated dams in this study. Also in a recent study, Hadrup et al demonstrated that Ag in both nano-particulate and ionic forms could affect brain neurotransmitter concentrations after intranasal exposure. Authors suggested that ionic Ag and Ag-NPs have similar neurotoxic effects (219). Further studies would be absolutely necessary for better understanding of Ag-NPs neurotoxicity mechanisms.

In conclusion, the results of present study showed that administration of Ag-NPs during gestation period did not cause reproductive/ developmental toxicity up to 20 mg/kg/day. However, results of this study suggested that Ag-NPs possibly transfer from dams to offspring through the placenta or minor pathway of milk. Also we showed that Ag in nanoparticulate and ionic forms may affect oxidative stress in both dams and pups, whereas in ionic form was more potent. Consequence of hippocampal sclerosis even in 0.2 mg/kg daily dose is alarming and should be considered in a risk assessment of Ag-NPs. Therefore, the NOAEL values reported in previous studies may be needed to adjust in the light of findings from this study.

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YEDİTEPE ÜNİVERSİTESİ

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ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
22.06.2012	270	15.06.2012 tarihli yazı	Prof.Dr.Ahmet AYDIN Doç.Dr.Ülkü ÜNDEĞER

'Nanopartiküllerin Toksikolojik Değerlendirmesi' başlıklı bilimsel araştırma Etik Kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oybirliği ile karar verilmiştir.
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