ความเป็นพิษของอนุภาคซิลเวอร์นาโนต่อเซลล์มะเร็งเยื่อบุปอดของมนุษย์ชนิดเอ 549

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TOXICITY OF SILVER NANOPARTICLES ON HUMAN LUNG CARCINOMA EPITHELIAL (A549) CELLS

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ปัจจุบันมีการใช้อนุภาคซิลเวอร์นาโน (AgNPs) ในผลิตภัณฑ์ทางการแพทย์และเครื่องอุปโภคหลายชนิด มีการศึกษาหลายการศึกษาแสดงให้เห็นว่า AgNPs ทำให้เกิดพิษต่อเซลล์หลายชนิดและกลไกการก่อพิษดังกล่าวมีความเกี่ยวข้องกับการสร้างอนุมูลออกซิเจนที่ว่องไว (ROS) งานวิจัยนี้มีวัตถุประสงค์ที่จะศึกษากลไกความเป็นพิษระดับชีววิทยาโมเลกุลของ AgNPs ต่อเซลล์มะเร็งเยื่อบุปอดของมนุษย์ชนิดเอ 549 รวมทั้งศึกษาความเป็นพิษของ AgNPs ภายใต้สภาวะที่มีการกำจัด ROS โดยการให้สาร N-acetyl cysteine (NAC) แก่เซลล์ก่อนสัมผัสกับ AgNPs จากการประเมินความเป็นพิษโดยวิธี MTT พบว่าเซลล์ที่ได้รับ AgNPs มีชีวิตรอดของลดลงอย่างมีนัยสำคัญ ($P < 0.05$) เมื่อความเข้มข้นและระยะเวลาที่สัมผัสกับ AgNPs เพิ่มขึ้น และ NAC สามารถเพิ่มการรอดชีวิตของเซลล์ได้อย่างมีนัยสำคัญ ($P < 0.05$) ในการทดลองเพื่อวัดระดับ ROS ภายในเซลล์พบว่า AgNPs ที่ความเข้มข้น 100 และ 200 µg/ml เหนี่ยวนำให้เกิดการสร้าง ROS เพิ่มขึ้นแตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ ($P < 0.05$) ซึ่ง NAC สามารถลดระดับ ROS นี้ได้ นอกจากนี้จากการศึกษาผลของ AgNPs ต่อ mitochondria membrane potential (MMP) ด้วยวิธี tetramethyl rhodamine ethyl ester (TMRE) ที่เวลา 24, 48 และ 72 ชม. พบว่า AgNPs ที่ความเข้มข้น 100 และ 200 µg/ml ทำให้ MMP ลดลงอย่างมีนัยสำคัญ ($P < 0.05$) และ NAC สามารถเพิ่มระดับ MMP ในกลุ่มที่ได้รับ AgNPs ที่ความเข้มข้น 100 µg/ml ได้ ส่วนการศึกษาผลของ AgNPs ต่อ cell cycle พบว่าเซลล์ที่ได้รับ AgNPs ที่ความเข้มข้น 100 และ 200 µg/ml มีการเพิ่มขึ้นของสัดส่วนเซลล์ใน subG1 และ S phase อย่างมีนัยสำคัญ ($P < 0.05$) และสัดส่วนเซลล์ใน subG1 ลดลงอย่างมีนัยสำคัญในกลุ่มที่ได้รับ NAC ($P < 0.05$) แต่ไม่พบความแตกต่างของสัดส่วนเซลล์ใน S phase ระหว่างกลุ่มที่ได้รับ และกลุ่มที่ไม่ได้รับ NAC เพื่อยืนยันผลดังกล่าวจึงทำการศึกษาการแสดงออกของโปรตีนที่เกี่ยวข้องกับ S phase ได้แก่ Proliferating nuclear antigen (PCNA) พบว่า AgNPs ลดแสดงออกของ PCNA อย่างเป็นสัดส่วนโดยตรงกับความเข้มข้นและ NAC ไม่สามารถเพิ่มการแสดงออกของ PCNA ได้ ผลการศึกษานี้แสดงให้เห็นว่ากลไกการก่อพิษของ AgNPs ต่อเซลล์มะเร็งเยื่อบุปอดของมนุษย์ชนิดเอ 549 มีทั้งแบบที่เกี่ยวข้องและไม่เกี่ยวข้องกับ ROS อย่างไรก็ตามกลไกการเกิดพิษของ AgNPs ยังต้องทำการศึกษาเพิ่มเติมอีกต่อไป

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PORNTIPA CHAIRUANGKITTI: TOXICITY OF SILVER NANOPARTICLES ON HUMAN LUNG CARCINOMA EPITHELIAL (A549) CELLS. ADVISOR: ASSOC. PROF. Pol.Lt.Col SOMSONG LAWANPRASERT, Ph.D., CO-ADVISOR : RAWIWAN MANIRATANACHOTE, Ph.D., CO-ADVISOR : ASST. PROF.PITHI CHANVORACHOTE, Ph.D., 89 pp.

Silver nanoparticles (AgNPs) are nowadays incorporated in a large number of consumer and medical products. Several experiments demonstrated that AgNPs cause toxicity to various cells via induction of reactive oxygen species (ROS). The present study was aimed to evaluate the mechanistic view of AgNPs' toxicity in A549 cells including the ROS-dependent and -independent pathways using a ROS scavenger, N-acetyl cysteine (NAC). Cytotoxicity test (MTT assay) showed that AgNPs significantly ($P < 0.05$) reduced cell viability in a concentration and time-dependent manner. AgNPs (100 and 200 μg/ml) significantly ($P < 0.05$) increased ROS formation which could be attenuated by NAC. Mitochondrial membrane potential (MMP), as measured by tetramethyl rhodamine ethyl ester (TMRE) assay at 24, 48 and 72 h was significantly ($P < 0.05$) reduced in AgNPs (100 and 200 μg/ml) treated groups in the concentration and time-dependent manner. NAC prevented the declination of MMP of AgNPs (100 μg/ml) treated group. Cell cycle analysis at 24, 48 and 72 h revealed the significant ($P < 0.05$) increment of subG1 and S phase population in treated cells and this subG1 ratio could be attenuated by NAC ($P < 0.05$). Interestingly, no statistical significant difference of S-phase population was observed between NAC-pretreated groups and that of the control. To provide mechanistic view of AgNPs-induced S phase arrest, the expression level of cell cycle-associated protein, proliferating nuclear antigen (PCNA) was investigated. PCNA expression was significantly ($P < 0.05$) concentration-dependent down-regulated by AgNPs and NAC could not prevent this effect. These observations allow us to envisage a possible scenario of AgNPs-induced cytotoxicity in A549 cells via both ROS-dependent and ROS-independent pathways. Experiments to elucidate mechanism of AgNPs-induced toxicity should be further performed.

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LIST OF ABBREVIATIONS

%	percent
^{o}C	degree Celsius
A549 cells	human lung carcinoma cells
ANOVA	analysis of variance
AgNPs	silver nanoparticles
CO_2	carbondioxide
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein diacetate
DI	deionized water
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
et al.	*et* alii, and other
FACS	Fluorescence Activated Cell Sorting
FBS	fetal bovine serum
g	gram (s)
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSH	glutathione
h	hour (s)
HRP	horseradish peroxidase
IgG	immunoglobulin G
µg	microgram (s)
µl	microliter (s)
µM	micromolar
mg	milligram (s)
min	minute (s)

ml	milliliter (s)
mM	millimolar
m^2/g	square meter (s) per gram
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl cysteine
NIH	National Institutes of Health
nm	nanometer (s)
PBS	phosphate-Buffered Saline
PCNA	proliferating cell nuclear antigen
PDI	polydispersity index
PI	propidium iodide
PVDF	polyvinylidene fluoride
RNase A	ribonuclease A
ROS	reactive oxygen species
SIN-1	3-morpholino-sydnonimine
S.D.	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM	transmission electron microscope
TMRE	tetramethyl rhodamine ethyl ester

CHAPTER I
INTRODUCTION

Nanotechnology is exponentially expanding and applied in various areas, such as health care, consumer products, medical preparations, electronic devices, food and feed, environmental health, and agriculture (Susan *et al.,* 2009). With reduction in size, specific surface area and reactivity of nanoparticles (NPs) dramatically increase. It has been reported that these novel physicochemical properties bring NPs to be bioreactive species when accessing into living organisms (Hussain *et al.,* 1994).

Silver nanoparticles (AgNPs) have gained considerable attention as compared to other metal nanomaterials because of their attractive physicochemical properties. AgNPs are nowadays incorporated in a large number of consumer and medical products (Chen *et al.,* 2008; Kamyshny *et al.,* 2005). AgNPs within the range of 20-50 nm demonstrated higher inhibiting efficacy on a wide range of bacteria and fungi than those of microsized particles (Bechert *et al.,* 2004; Kim *et al.,* 2007). AgNPs have been applied in the treatment of the immunologic and inflammatory diseases for years (Shin *et al.,* 2007). Estimated from 800 nano-consumer products, about 30% are claimed to contain AgNPs (Villalobos-Hernandez and Muller-Goymann *et al.,* 2004). Despite the rapidly growing sector of AgNPs contained products on the market, studies addressing their toxicity concerning the human health and environmental impact are comparatively few.

Presently, several experiments have demonstrated that nano-sized silver can be toxic to human's vital organs especially lung. AgNPs not only bind proteins contained –SH groups with strong affinity but also persistently accumulate in the lung for many years (Hussain *et al.,* 1994). Furthermore, it has been proposed that major mechanism of AgNPs-mediated cytotoxicity is the induction of reactive oxygen species (ROS) generation (Carlson *et al.,* 2008a; Carlson *et al.,* 2008b). Supporting this assumption, the increase of ROS levels accompanied by the decrease of antioxidant enzymes such as, glutathione peroxidase and superoxide dismutase as well as the increase of lipid peroxidation and DNA adducts formation have been detected after exposure to PVP-coated AgNPs (Foldbjerg *et al.,* 2010). In additions, the correlation between ROS

generation and indicators of cytotoxicity such as DNA damage, apoptosis and necrosis have been reported (Kim *et al.,* 2009). Interestingly, the cytotoxicity of AgNPs was greatly decreased by pretreatment with the ROS scavenger, N-acetyl-cysteine (NAC) (Foldbjerg *et al.,* 2010).

Another mechanism of AgNPs-mediated cytotoxicity is mitochondrial-related toxicity. Generally, mitochondrial are sensitive to many toxic substances and closely related to oxidative balance in cells. Impairment of mitochondrial function results in drastic consequences on redox potential equilibrium and normal cell function. Several studies have demonstrated that mitochondria are the major targets for phagocytosed particles (Kamp *et al.,* 2002; Xia *et al.,* 2006). They are considered as major cellular compartments relevant for nanoparticles toxicity (Unfried *et al.,* 2007). Nanoparticles potentially damage mitochondrial structure through oxidative stress (Pan *et al.,* 2009; Sun *et al.,* 2011). As a result of structural damage, the loss of mitochondrial membrane integrity, opening of the permeability transition pore (PTP), ROS production and cell death may occur (Li *et al.,* 2003; Xia *et al.,* 2007). Experimentally, particles initiate cytotoxicity by mitochondrial perturbation leading to increase of ROS production, decrease of mitochondrial membrane potential and induction of apoptosis (Upadhyay *et al.,* 2003). Furthermore, recent study has shown negatively dose-response in mitochondrial depolarization following exposure to metal oxide and silver nanoparticles (Hanna *et al.,* 2009; Hussain *et al.,* 2005). Presumably, NAC can prevent silica nanoparticles-induced mitochondrial depolarization (Xin *et al.,* 2010).

Apart from cytotoxicity, disturbance of normal cell cycle is one of the effects that can be observed in AgNPs treated cells. Regarding the cell cycles, AgNPs induced G1 and S phase arrest in a mouse peritoneal macrophage cell line (RAW264.7) (Park *et al.,* 2010). There have been reported that starch-coated silver NPs induced G2/M phase arrest and DNA damage in human glioblastoma cells and human lung fibroblasts (AshaRani *et al.,* 2009). Mainly compounds which contain anti-DNA synthesis effect can perturb S phase progression (Black *et al.,* 1989). However, little is known about effect of AgNPs on each phase of cell cycle. According to National Institutes of Health (NIH) established assays for proliferative effects of nanoparticles, immunostaining of proliferating cell nuclear antigen (PCNA)

correlates well with various methods (Jedd *et al.,* 2010). PCNA, a protein synthesized in the early G1 and S phases of the cell cycle, is suggested to function in cell cycle regulation, DNA replication and DNA repair (Maga and Hübscher, 2003). PCNA down-regulation may indicate cell cycle arrest (Engel *et al.,* 2003). Therefore, analysis of PCNA expression could provide some mechanistic views of nanoparticles-induced cell cycle arrest.

Compared to other routes of AgNPs exposure, lung is the least protective area. For this reason, consequences of pulmonary exposure to AgNPs should be carefully investigated. While the particle-induced ROS generation and oxidative injury has been widely accepted as a vital mechanistic paradigm for AgNPs toxicity, other patterns of injury such as DNA damage, mitochondrial injury, changes of normal cell cycle should be concerned. Thus, the aim of this study was to investigate cell viability, intracellular ROS, change of mitochondrial membrane potential and changes of cell cycle in response to AgNPs treatment by using the A549 cells as a model in order to elucidate possible mechanisms of AgNPs-induced pulmonary toxicity.

Objective

This study is performed to investigate possible mechanisms of AgNP-induced toxicity in A549 cells.

Hypothesis

AgNPs exert toxicity in A549 cells by two major pathways namely, ROS-dependent and ROS-independent pathways. The overall effects of AgNPs on A549 cells include initiation of oxidative stress in cells, change of mitochondrial membrane potential and disturbance of normal cell cycle.

Benefit gained from this study

This study provides the toxicological effects and possible mechanisms of AgNP-induced toxicity on A549 cells.

CHAPTER II
LITERATURE REVIEWS

1. Nanoparticles (NPs)

The rapid advancement of nanotechnology leads to production of a vast array of engineered nanoparticles (Figure 2) which are defined as materials measured in length or diameter of less than 100 nm (10^{-9} m) in at least one dimension (Figure 1). The U.S. National Nanotechnology Institute categorizes four generations of nanotechnology. Firstly, in 2006 was the era of materials which were designed to perform only one task named passive nanostructures. Secondly, form 2006 to 2010 active nanostructures for multitasking purposes were introduced, for example, actuators, drug delivery devices, and sensors. Thirdly, from the second phase up to now, feature nanosystems with thousands of interacting components has been initiated. A few years from now, the integrated nanosystems, functioning like a mammalian cell with hierarchical systems within systems will be generated. Nowadays, nanoparticles are exponentially expanding and being applied in various areas such as health care and consumer products, medical preparations for the purposes of diagnosis, imaging, and drug delivery, electronic devices, food and supplements, environmental health, and agriculture (Susan *et al.,* 2009). For instance, aluminium for propulsion devices, sunscreens, cosmetics, paints, and fuel additives, Quantum Dots for drug carriers, fluorophores and silver for bio-imaging and bactericidal agents. Interestingly, in environmental aspect, nano-sized sensors are considered as sensitive detector in tracking of contaminants (Villalobos-Hernandez and Muller-Goymann *et al.,* 2004).

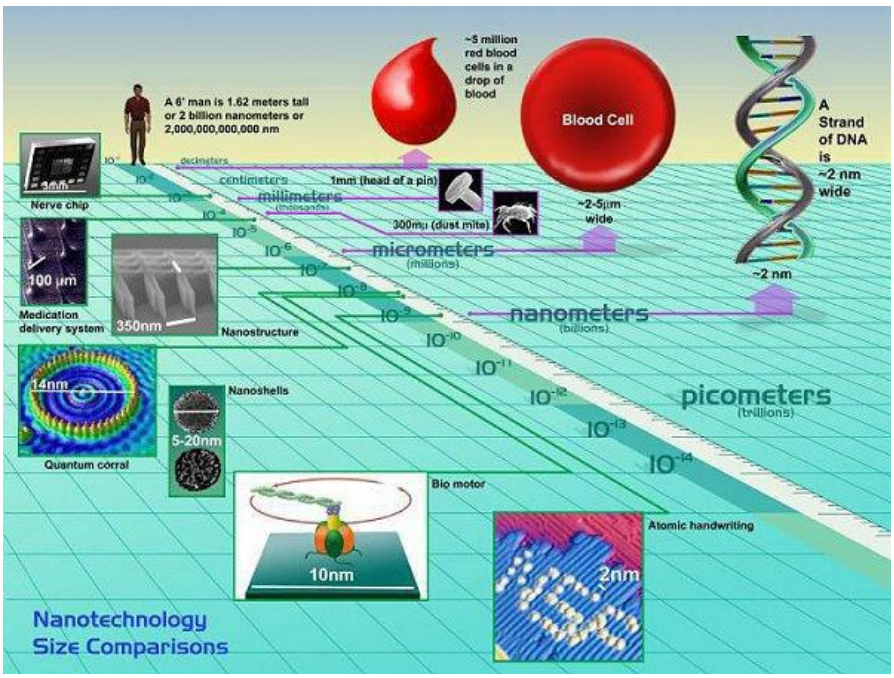


Figure 1 Size comparison of nanoparticles to household and scientific objects (Graffin *et al.,* 2008)

2. Toxicity of Nanoparticles

After exposure, NPs readily distribute throughout the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria, and finally trigger injurious responses. Recent studies have shown that NPs can pass through cell membranes and biological barriers such as the blood-brain barrier, deposit in target organs, and exert effects on functions of biological system (Foley *et al.,* 2002; Kim *et al.,* 2006). Furthermore, NPs potentially raise autoimmunity by acting like haptens to modify cellular protein structures which can induce immune responses.

The unique toxicities of NPs depend on their small size (surface area and size distribution), chemical composition (purity, crystallinity, electronic properties), surface modifying (surface reactivity, surface groups, coating materials), unique shape, and dimension. The combination of these specific attributes can potentially generate biological adverse effects in living cells that would not be detectable from the bulk material.

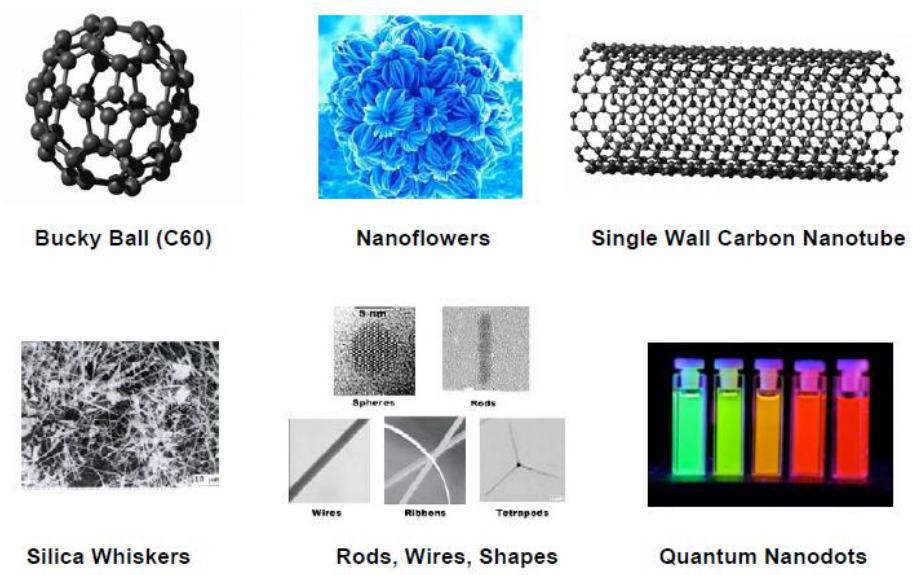


Figure 2 Various types of nanoparticles (Cristina *et al.,* 2007)

The main characteristic of NPs is their size, which falls in the transitional zone between individual atoms or molecules and the corresponding bulk materials. This smaller size can modify the physicochemical properties of the material including dramatic increases of specific surface area and reactivity of NPs (Figure 3) (Nel *et al.,* 2005). This tiny characteristic creates the opportunity for increased uptake and interaction with biological tissues. Experimentally, the higher surface area per mass NPs possess, the larger amount of atoms interact with their surroundings especially cell membrane (Bene *et al.,* 2005). *In vivo* study investigating the correlations between lung injury and particle size of TiO_2 particles of two particle sizes (20 nm, ultrafine; 250 nm, fine) of the same crystalline structure (anatase) showed that ultrafine particles can induce pulmonary inflammation, oxidative stress, and distal organ involvement more than the larger ones (Oberdörster *et al.,* 2005b). Besides, toxicity study of AgNPs showed that significant increase of intracellular ROS was induced by Ag-15 nm compared to the Ag-55 nm treated groups (Carlson *et al.,* 2008). *In vitro* toxicological study of NPs in BRL 3A rat liver cells demonstrated that the most smallest particles tended to cause more harm to cells than the larger ones

(Hussain *et al.,* 2005). It is apparently supposed that the unique toxicities of NPs attribute to their size.

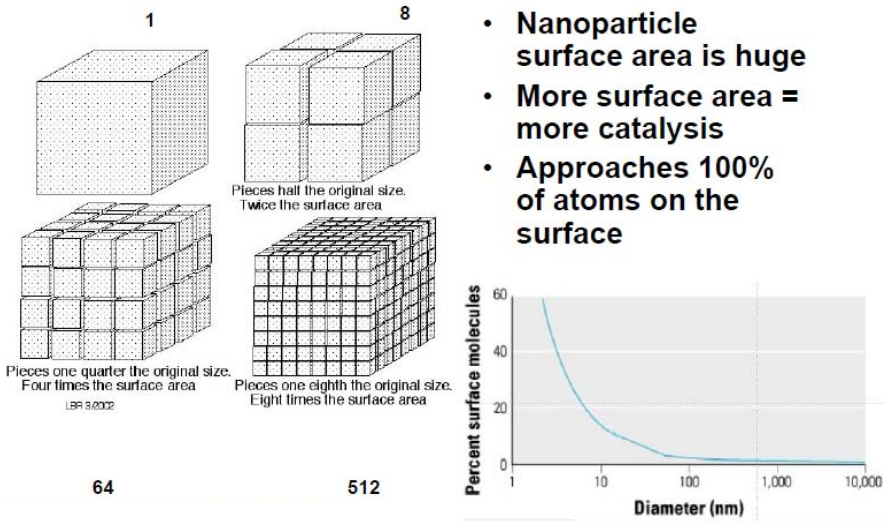


Figure 3 Inverse relationship between particle size and number of surface area (Bruce *et al.,* 2007)

Additionally, manufactured NPs have been designed to possess unique physical and chemical attributes such as shape, crystallinity, surface charge, surface coating, elemental composition and solubility in order to meet various industrial requirements. Particle coating, surface treatments, surface excitation by ultraviolet (UV) radiation, and particle aggregation can modify the toxicities of particles. Some NPs may possibly exert their toxic effects as aggregates or through the release of toxic chemicals. These physicochemical properties bring NPs to bio-reactive species when accessing into living organisms and its typical toxicity was considered from these varied attributes (Hussain *et al.,* 1994). In summary, the same metal with different attributes may pose threat to cells via different mechanisms. For instances, a study has demonstrated that the structure of NPs (TiO_2 in different forms such as, anatase and rutile forms) also plays a key role in toxicity (Warheit *et al.,* 2006). Moreover, cell membrane penetration ability may vary from type of surface coating materials. (Verma *et al.,* 2008). As mentioned above, the biological impacts and biokinetics of NPs rely on various factors because these parameters can modify cellular uptake,

protein binding, translocation of NPs to the target site, and the possibility of causing tissue injury (Oberdörster *et al.*, 2005b). At the target site, NPs may trigger tissue injury by one or more mechanisms (Table 1).

Table 1 Table 2. NM effects as the basis for pathophysiology and toxicity. Effects supported by limited experimental evidence are marked with asterisks; effects supported by limited clinical evidence are marked with daggers. 2005)

Experimental NM effects	Possible pathophysiological outcomes
ROS generation*	Protein, DNA and membrane injury,* oxidative stress†
Oxidative stress*	Phase II enzyme induction, inflammation,† mitochondrial perturbation*
Mitochondrial perturbation*	Inner membrane damage,* permeability transition (PT) pore opening,* energy failure,* apoptosis,* apo-necrosis, cytotoxicity
Inflammation*	Tissue infiltration with inflammatory cells,† fibrosis,† granulomas,† atherogenesis,† acute phase protein expression (e.g., C-reactive protein)
Uptake by reticulo-endothelial system*	Asymptomatic sequestration and storage in liver,* spleen, lymph nodes,† possible organ enlargement and dysfunction
Protein denaturation, degradation*	Loss of enzyme activity,* auto-antigenicity
Nuclear uptake*	DNA damage, nucleoprotein clumping,* autoantigens
Uptake in neuronal tissue*	Brain and peripheral nervous system injury
Perturbation of phagocytic function,* "particle overload," mediator release*	Chronic inflammation,† fibrosis,† granulomas,† interference in clearance of infectious agents†
Endothelial dysfunction, effects on blood clotting*	Atherogenesis,* thrombosis,* stroke, myocardial infarction
Generation of neoantigens, breakdown in immune tolerance	Autoimmunity, adjuvant effects
Altered cell cycle regulation	Proliferation, cell cycle arrest, senescence
DNA damage	Mutagenesis, metaplasia, carcinogenesis

(*) Effects supported by limited experimental evidence

(†) Effects supported by limited clinical evidence

Main exposure routes of NPs including gastrointestinal tract (GIT), skin, lung, and circulatory system. In case of binding to proteins, NPs could possibly accelerate protein degradation leading to structural or functional changes. When NPs reaching to cells, they will be reacted with defensive enzymes which eliminate, sequester, or dissolve them, such as antioxidant enzymes that deal with ROS generated from NPs.

All considered, the available data confirm that ROS play an important role in mechanisms of NPs-induced cytotoxicity because most NPs which exert cytotoxicity can produce ROS (Xia *et al.*, 2006). Consequently, ROS bring about pro-oxidant

environment in the cells, perturb a redox potential equilibrium and lead to adverse biological consequences ranging from early stage of inflammation to serious cell death (Figure 4) (Nel *et al.*, 2005; Maria *et al.*, 2007; Kim *et al.*, 2009). Supporting this assumption, the cytotoxicity of NPs was greatly decreased by pretreatment with the ROS scavenger, N-acetyl-cysteine (NAC) (Foldbjerg *et al.*, 2010).

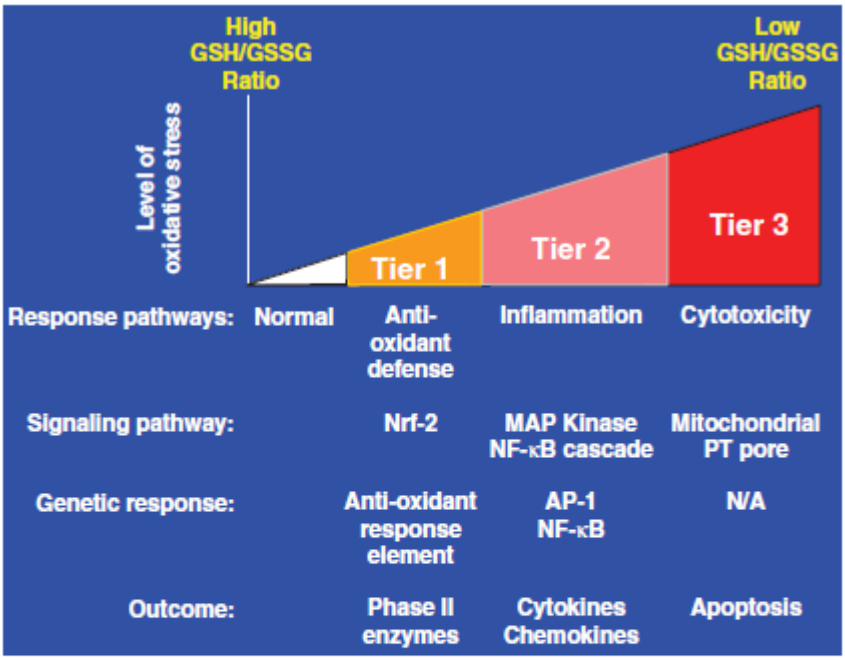


Fig. 3. The hierarchical oxidative stress model. At a lower amount of oxidative stress (tier 1), phase II antioxidant enzymes are induced via transcriptional activation of the antioxidant response element by Nrf-2 to restore cellular redox homeostasis. At an intermediate amount of oxidative stress (tier 2), activation of the MAPK and NF-κB cascades induces pro-inflammatory responses. At a high amount of oxidative stress (tier 3), perturbation of the mitochondrial PT pore and disruption of electron transfer results in cellular apoptosis or necrosis. [Adapted from (*11*)] N/A means not applicable.

Figure 4 The hierarchical oxidative stress model (Nel *et al.*, 2005).

3. Nanoparticles-induced pulmonary toxicity

3.1 Anatomy of the Lung

Lung is a crucial organ of respiratory system. Its structure consists of two general regions called the upper and lower airways. Air in or out of the lungs is transportated from the upper to the lower airway. Major function of lower airway is gas exchange to supply cellular oxygen demand. Most of inhaled particles between 5-30 μm can easily reach the nasopharyngeal region and be expelled by reflex mechanism such as coughing etc. Particles between 1-5 μm can enter into the trachea, bronchial and bronchiolar region. Mucociliary escalator is responsible for clearance these micron sized particles. Finally, particles smaller than 1 μm can access to the deep regions: alveolar sacs (Figure 5). Accordingly, it can be said that foreign particles deposition depends greatly, but not inclusively, on size. At alveolar sac region, particles are usually phagocytosed by pulmonary alveolar macrophages where the alveolar sacs and alveolar epithelium reside. Thus, the alveolar sacs and alveolar epithelium are the main targets for NPs.

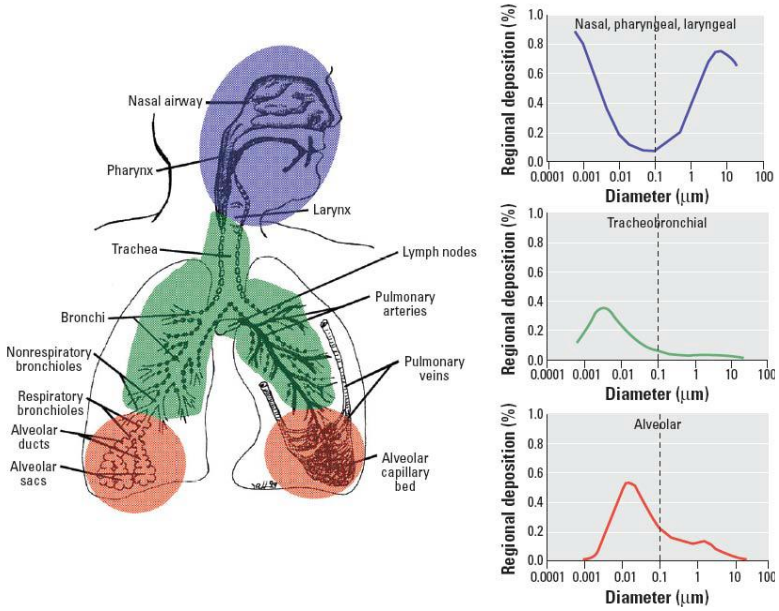


Figure 5 Relationship between particles size and area of deposition (Kleinstreuer *et al.*, 2009)

Owing to the large surface area and the least protective barrier, lung is considered as a vulnerable organ for NPs invasion which is directly contact with the atmospheric NPs. Several defense mechanisms, including mucociliary escalator transport and phagocytosis by macrophages, keep the mucosal surfaces free from deposited particles. However, it is quite hard for lung to be effective in getting rid of NPs. For examples, the small size of NPs allows them to easily penetrate into the deep air spaces of the alveoli and be readily taken up by lung epithelial cells and fibroblasts. Another susceptible factor of lung to NPs is single-cell layer which makes it a poor barrier against the entry of NPs from the alveolar to the blood circulation. Entry of NPs across the alveolar–capillary barrier into the circulatory system are regulated by clathrin-coated pits and caveolae (Gumbleton *et al.,* 2001). Caveolae, a structure abundantly expressed on lung capillaries and type l alveolar cells, are the parts of the plasma membrane full with caveolin-1. It has been hypothesized that inspiratory expansion and expiratory contraction of lung alveoli may lead to the opening and closing of the caveolae to assist macromolecular transport across the alveolar membrane.

After NPs approaching to respiratory system, the translocation to other organs rapidly occurs (Figure 6). Some demonstrated that gold nanoparticles (AuNPs) and carbon-based NPs are capable of penetrating cellular membranes rapidly then could potentially overcome the blood–brain barrier and translocate to the brain by the uptake of these NPs through the olfactory bulb (Oberdörster *et al.,* 2004). Furthermore, several *in vitro* studies show that NPs induce oxidative stress and mitochondrial damage via penetration into the lung epithelial cells and exhibit more potent cytotoxicity than the larger particles. The lung is not only an entrance for NPs but also a by-pass of NPs to the circulatory system. For this reason, NPs from lungs can translocate to other organs quite readily. Experimentally, AuNPs being into rats were detected in other organs a few weeks later (Yu *et al.,* 2007). Therefore, it seem that the smaller size of the particles, the wider bio-distribution in the body and the longer the bio-persistence will occur resulting in the greater impact of toxicity in cells, tissues and organ systems.

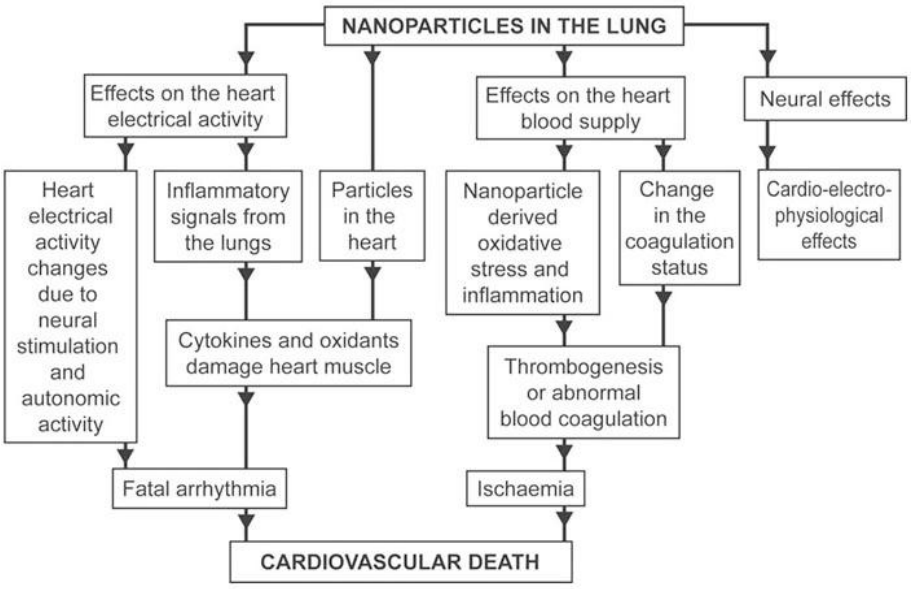


Figure 6 Effect of inhaled nanoparticles on other organs

(Cristina *et al.*, 2007)

3.2 Lung disorders resulting from NPs exposure

3.2.1 Exacerbation of asthma

Asthma is an inflammatory disease of the pulmonary airways. Antigen induces lung hypersensitivity leading to the various symptoms such as wheezing etc. In general, asthmatic patients are more susceptible to NP-induced pulmonary disorder than healthy people. Several studies have shown that fine particles could deposit and accumulate in the lungs of including patients with chronic obstructive lung disease (COPD) (Daigle *et al.,* 2003). It is apparently that vulnerable people would express lung disorder because of their greater NP deposition than normal people.

3.2.2 Pulmonary fibrosis

Pulmonary fibrosis is the case of tissue hyper-reactivity to antigen. Subsequently, fibrous connective tissue was generated and expanded. That leads to the distortion of lung architecture, inducing bronchiectasis and chronic respiratory infection and causing hypoxemia and pulmonary hypertension (Huaux *et al.,* 2007). Lately, it is evident that the presence of multiwall-carbon nanotubes (MWCNTs) in the subpleural region in lungs of mice gives rise to fibrosis and scarring (Ryman-Rasmussen *et al.,* 2009).

3.2.3 Pneumoconiosis

A widely known occupational related-pulmonary disorder, Pneumoconiosis is classified into two types, fibrotic and non-fibrotic. Differences of both clinical conditions are affected by a multitude of particle types, size and dose of inhaled NPs. According to the hypothesis, some NPs such as carbon nanotubes could behave like asbestos to cause this pathological damage to lung. Previuos study

demonstrated pathogenic effect of MWCNTs which is like asbestos-induced lung disorder in mice (Poland *et al.,* 2008). To illustrate the effect of NPs in the lung, the mechanisms of NPs-related pulmonary toxicity at the cellular level should be provided.

3.3 Mechanistic views of NPs-induced pulmonary toxicity

3.3.1 Ineffective clearance of NPs

The epithelium of respiratory tract is covered with epithelial lining fluid composed of various neutralizing agents. Surfactant agents are the crucial components. Their vital function is associated with the displacement of NPs which facilitate mucociliary clearance. Furthermore, efficiently opsonization requires the proteins in surfactant (Schurch *et al.,* 1990).

In additions, NPs are easily agglomerated and phagocytosed by the alveolar macrophages (Geiser *et al.,* 2003). Consequently, alveolar macrophages which are activated can release oxygen radicals, proteolytic enzymes, proinflammatory mediators and growth-regulating proteins leading to lung inflammation (Oberdörster *et al.,* 2001). Besides, studies have elicited the production of proinflammatory mediators that attract leukocytes and upregulate adhesion molecules on alveolar cell surfaces (Fuji *et al.,* 2001).

3.3.2 Oxidative stress

NPs are well-known to initiate reactive oxygen species (ROS), which could lead to oxidative stress (Figure 7). ROS generation by NPs could depend on three main factors (Knaapen *et al.,* 2004) as following: active redox cycling on the surface of NPs, particularly the metal-based NPs, oxidative groups functionalized on NPs and particle–cell interactions. For the particle–cell interactions, it is a prominent factor in lungs where there is a rich pool of ROS producers like the inflammatory phagocytes, neutrophils and macrophages. Excessive production of ROS activates a

cytokine cascades, such as interleukins (IL) and tumor necrosis factor (TNF). Studies have shown that TiO_2NPs induced the elevation of proinflammatory cytokines, such as IL-1, TNF-α, IL-6, macrophage inhibitory protein in NPs treated lungs (Park *et al.*, 2009).

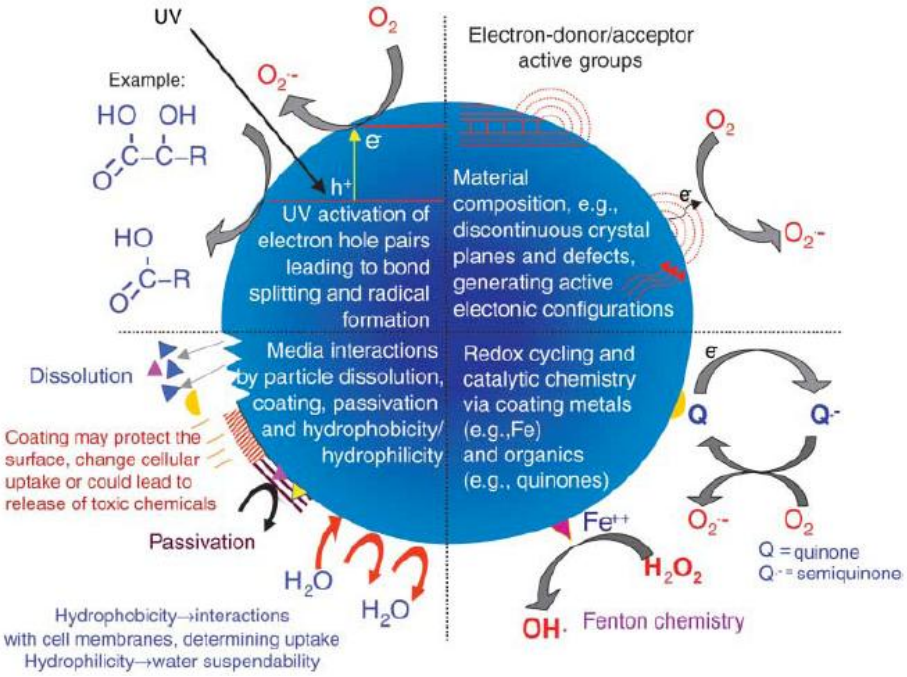


Figure 7 Possible mechanisms of ROS-dependent NPs toxicity (Nel *et al.*, 2005).

3.3.3 Activation of inflammation processes

Inflammation appears to be the most common of NPs toxicities. Oxidative stress is the major molecular mechanism giving rise to inflammation responses by NPs. Research on TiO_2NPs show they can potentially induce pulmonary neutrophilia via increased production of neutrophil-attracting chemokines CXCL1, TNF-α and IL-8 (Figure 8) (Fuji *et al.,* 2001) accompanied by eosinophils and lymphocytes in the bronchoalveolar lavage fluid (Ganguly *et al.,* 2009).

3.3.4 Genotoxicity

NPs-induced genotoxicity and carcinogenesis are quite well-supposed hypothesis. Several *in vivo* experiments have pointed out that long-term inflammation and oxidative stress present in tissue environment eventually induces DNA damage in cells and tissues. Continuous generation of oxidative environment in the cell causes gene mutations or deletions leading to larger-scale mutagenesis and carcinogenicity and subsequently development of tumors and cancer (Singh *et al.,* 2009). Regarding the DNA damaging properties of certain classes of NPs, metal-based NPs like AgNPs are in this issue (AshaRani *et al.,* 2009). Exposure to NPs in long-term, displayed genome instability, altered cell cycle and induced protein expression of p53 and DNA repair-related proteins (Mroz *et al.,* 2008).

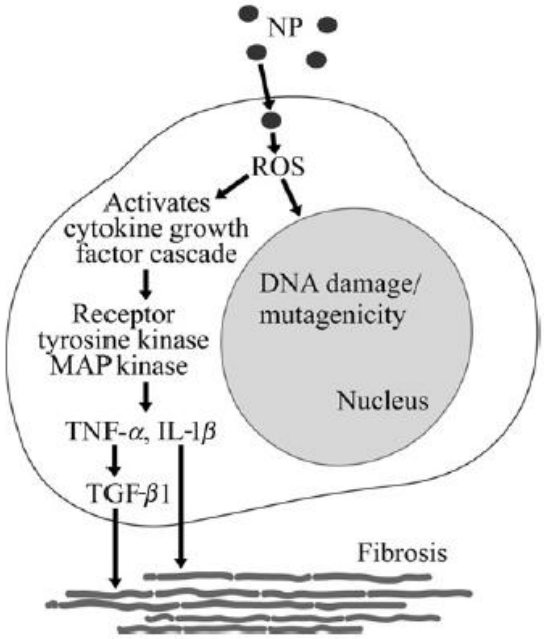


Figure 3 Possible mechanistic pathway for pulmonary toxicity induced by exposure to NPs. Exposure to NPs may lead to oxidative stress due to increased production of ROS and downstream signaling responses that promote fibrosis and produce genotoxicity. NP, nanoparticles; ROS, reactive oxygen species

Figure 8 Possible mechanistic pathways for pulmonary toxicity induced by NPs exposure (Fuji *et al.,* 2001)

4. Silver nanoparticles (AgNPs)

AgNPs have gained considerable attention as compared to other metal nanomaterials because of their attractive physicochemical properties. AgNPs are nowadays incorporated in a large number of consumer and medical products (Chen *et al.,* 2008; Kamyshny *et al.,* 2005). Due to its effectively antimicrobial activity and safety profile, nano-silver has been introduced to be a therapeutic agent for a long time specifically in the management of open wounds and burns.

To produce AgNPs from metallic silver, several methods including spark discharging, electrochemical reduction, solution irradiation and cryochemical synthesis have been applied. (Chen *et al.,* 2008). Most AgNPs derived from these methods are smaller than 100 nm in diameter and consist of approximately 20-15,000 silver atoms depending on their size. (Oberdörster *et al.,* 2005a) AgNPs may have different shapes, such as spheres, rods, cubes. At the nano level, the AgNPs exhibit deviating physico-chemical properties (like pH-dependent partitioning to solid and

dissolved particulate matter) and biological activities compared with the bulk materials (Lok *et al.,* 2007). The higher surface area per mass resulting from the nano-size yields much more possible interaction between AgNPs and their surroundings.

4.1 Uses of Silver nanoparticles

AgNPs within the range of 20-50 nm demonstrated higher inhibiting efficacy on a wide range of bacteria and fungi than those of microsized particles (Bechert *et al.,* 2004; Kim *et al.,* 2007). In detail, AgNPs contain effective anti-bacterial properties including a broad spectrum killing against Gram-negative, Gram-positive bacteria and antibiotic-resistant strains. Gram-negative bacteria, such as *Acinetobacter, Escherichia, Pseudomonas, Salmonella* and *Vibrio*. Gram-positive bacteria include *Bacillus, Clostridium, Enterococcus, Listeria, Staphylococcus,* and *Streptococcus*. Besides, antibiotic-resistant bacteria include methicillin-resistant and vancomycin-resistant *Staphylococcus aureus,* and *Enterococcus faecium* (Burrell *et al.,* 1999; Yin *et al.,* 1999; Percival *et al.* 2007). The enhancement of antibacterial activity was detected when co-treatment of AgNPs and various antibiotics such as *penicillin G, amoxicillin, erythromycin, clindamycin,* and *vancomycin* (Shahverdi *et al.,* 2007). In additions, size-dependent antimicrobial activity of AgNPs has been reported in Gram-negative bacteria and Gram-positive bacteria (Morones *et al.,* 2005; Panacek *et al.* 2006). Moreover, shape-dependent antimicrobial activity of AgNPs conducted with Gram-negative bacteria has been shown that truncated triangular silver nanoplates were found to display the strongest anti-bacterial activity (Pal *et al.,* 2007). Many researches have shown that AgNPs anchor and penetrate to the cell wall of Gram-negative bacteria (Morones *et al.,* 2005). By this way, AgNPs could disturb normal cell permeability leading to an uncontrolled transport through the cytoplasmic membrane and ultimately cell death. Also, it has been proposed that this mechanism is related to the formation of free radicals and subsequent free radical-induced membrane damage. (Danilczuk *et al.,* 2006). Free radicals or/and AgNPs themselves could possibly interact with DNA and initiate DNA damage. This may disturb cell division, DNA replication ultimately leading to cell death. Molecular mechanism studies have pointed out that AgNPs may modulate the phosphotyrosine bacterial

peptides that could affect intracellular signaling and therefore inhibit the growth of bacteria (Shrivastava *et al.,* 2007).

In accordance with great antimicrobial activity, antifungal and antiviral properties have been reported. AgNPs are the broad spectrum and fast-acting fungicidal agents against *Aspergillus, Candida and Saccharomyces* (Wright *et al.,* 1999). Furthermore, AgNPs inhibit HIV-1 virus replication (Sun *et al.* 2005).

From the ancient time, AgNPs have been applied in treatment of immunologic and inflammatory diseases. (Shin *et al.,* 2007). Thus, the anti-inflammatory properties are widely interested. In animal models, AgNPs could alter the expression of matrix metallo-proteinases, important proteolytic enzymes in inflammatory and repair processes. Furthermore, AgNPs could suppress the expression of TNF-α, IL-12, and IL-1β, and induces apoptosis of inflammatory cells (Bhol and Schechter, 2007). Moreover, AgNPs modulate cytokines involved in wound healing (Tian *et al.,* 2007).

4.2 Human exposure

4.2.1 Exposure via food

The wide use of AgNPs in the food production chain includes the processing, conservation and consumption (Table 2). For food supplements, AgNPs have been claimed to purify some organs, support the immune functions and alleviate some severe illness. However, these statements have not been proved yet. As mentioned above, consumer exposure is expected to be high when nanoparticles are used as supplements. Nevertheless, the major sector using AgNPs is coatings process to prevent bacterial growth. So the actual exposure of humans is hard to estimate.

Table 2 Summary of applications of AgNPs in the food production chain (Susan *et al.,* 2009)

Chain phase	Application	Nanotechnology	Function
Processing of food	Food preparation equipment	Incorporated nanosized silver particles	Anti-bacterial coating of food handling devices
Conservation	Refrigerators Storage containers	Incorporated nanosized silver particles	Anti-bacterial coating of storage devices
	Food products	Nanosized silver sprays	Antibacterial action
	Packaging materials	Incorporation of active nano-silver particles	Oxygen scavenging, prevention of growth of bacteria
Food consumption	Supplements	Colloidal metal nanoparticles	Claimed to enhance desirable uptake

4.2.2 Exposure via consumer products

Several nanomaterials are used in consumer products such as titanium dioxide and zinc oxide in skin care preparations and organic nanomaterials such as nanovitamins (Dekkers *et al.,* 2007). However, the highest number of nanomaterial used in various products at this moment appears to be AgNPs. Estimated from 800 nano-consumer products, about 30% are claimed to contain AgNPs. (Villalobos-Hernandez and Muller-Goymann *et al.,* 2004). Another sectors, nanosilver (50 nm) can enhance desirous property of ink used in printing on various surfaces, such as glass (Kamyshny *et al.,* 2005; Magdassi *et al.,* 2003). AgNPs have also been used as purificant in drinking and swimming pool water (Chen *et al.,* 2008). The consumer product containing AgNPs can be categorized into five categories including electronics, filtration, purification, neutralization, sanitization, personal care and cosmetics, household products/home improvement and textile and shoes.

4.2.3 Exposure via medical products

Due to their antimicrobial properties, AgNPs have been introduced in curative and preventive medicine. Silver coating of medical devices can solve bacterial-derived problems, such as bacterial adhesion, colonization, biofilm formation and human-derived problems, such as adhesion of glycoproteins from tissue and blood plasma. Other potential applications of medical devices containing AgNPs are infusion ports, orthopedic protruding fixation devices, endovascular stents,

urological stents, endoscopes, electrodes, peritoneal dialysis devices, subcutaneous cuffs, surgical and dental instruments (Table 3).

Table 3 Medical uses of AgNPs (Lee *et al.*, 2003)

Medical domains	Examples
Anesthesiology	Coating of breathing mask Coating of endotracheal tube for mechanical ventilatory support
Cardiology	Coating of driveline for ventricular assist devices Coating of central venous catheter for monitoring
Dentistry	Additive in polymerizable dental materials Silver-loaded SiO_2 nanocomposite resin filler
Diagnostics	Nano-silver pyramids for enhanced biodetection Ultrasensitive and ultrafast platform for clinical assays for diagnosis of myocardial infarction Fluorescence-based RNA sensing Magnetic core/shell Fe_3O_4/Au/Ag nanoparticles with tunable plasmonic properties
Drug delivery	Remote laser light-induced opening of microcapsules
Eye care	Coating of contact lens
Imaging	Silver/dendrimer nanocomposite for cell labelling Fluorescent core-shell $Ag@SiO_2$ nanoballs for cellular imaging Molecular imaging of cancer cells
Neurosurgery	Coating of catheter for cerebrospinal fluid drainage
Orthopedics	Additive in bone cement Implantable material using clay-layers with starch-stabilized silver nanoparticles Coating of intramedullary nail for long bone fractures Coating of implant for joint replacement Orthopedic stockings
Patient care	Superabsorbent hydrogel for incontinence material
Pharmaceutics	Treatment of dermatitis Inhibition of HIV-1 replication Treatment of ulcerative colitis Treatment of acne
Surgery	Coating of hospital textile (surgical gowns, face mask)
Urology	Coating of surgical mesh for pelvic reconstruction
Wound care	Hydrogel for wound dressing

5. Toxicity of silver nanoparticles

5.1 In vitro studies

Presently, several experiments have demonstrated that AgNPs can be toxic to human's vital organs such as lung where AgNPs are inhaled, liver where AgNPs are extensively metabolized and kidney where AgNPs are mainly excreated.

There are various *in vitro* studies on the effects of AgNPs varying size between 1 and 100 nm. AgNPs not only bind proteins contained –SH groups with strong affinity but also persistently accumulate in the lung for a long time (Hussain *et al.,* 1994). Later, they evaluated the toxicity of several nanoparticles, including nano-silver (15 and 100 nm) on a rat liver derived cell line, BRL 3A. After exposure to AgNPs for 24 h, mitochondrial function and membrane integrity were significantly decreased. Microscopic evaluation displayed that AgNPs remained in cells and associated with membranes. They concluded that observed cytotoxicity was attributed to AgNPs-mediated oxidative stress, as indicated by the detection of GSH depletion, reduced mitochondrial potential and increased ROS levels. At the same time, a concentration-dependent effect on mitochondrial function, cell viability and membrane integrity (LDH leakage) were comfirmed (Braydich-Stolle *et al.,* 2005). Also, inhibition of dopamine production at neuroendocrine cells was demonstrated by AgNPs (Hussain *et al.,* 2006). The decrease of glutathione peroxidase and superoxide dismutase as well as the increase of lipid peroxidation and DNA adducts formation have been detected after exposure to starch-coated and PVP-coated AgNPs (Arora *et al.,* 2008; Foldbjerg *et al.,* 2010). The correlation study between ROS formation and indicators of cytotoxicity such as DNA damage, apoptosis and necrosis demonstrated the correlation between toxic effects of AgNPs and intracellular ROS level (Kim *et al.,* 2009). Nanoparticles potentially damage mitochondrial structure through oxidative stress (Pan *et al.,* 2009; Sun *et al.,* 2011). As a result of structural damage, the loss of mitochondrial membrane integrity, opening of the permeability transition pore (PTP), ROS production and cell death may occur (Li *et al.,* 2003; Xia *et al.,* 2007). Experimentally, particles initiate cytotoxicity by mitochondrial perturbation leading to increase of ROS production, decrease of mitochondrial membrane potential

and induction of apoptosis (Upadhyay *et al.,* 2003). Regarding toxicity to cell proliferation, starch-coated silver NPs could induce G2/M phase arrest and DNA damage in human glioblastoma cells and human fibroblasts (AshaRani *et al.,* 2009) Also, AgNPs induced G1 and S phase arrest (Park *et* al., 2010). On the contrary, preparation containing 1.0% of AgNPs (5-50 nm) in bone cement, a dose at which antibactericidal activity was seen, did not cause cytotoxicity towards mouse fibroblasts cells and human osteoblast cell line (Alt *et al.,* 2004).

5.2 Animal studies

After intravenous administration, rats exposed to high doses of colloidal silver died from lung oedema while liver, spleen and kidney showed signs of toxicity and calculated LD50 was about 67 mg/kg (Schmaehl and Steinhoff, 1960). Recently, the 90-day inhalation toxicity study performed in rats inhaled AgNPs 6 h/day at different concentration in atmosphere. They did show the decrease of lung function including tidal volume, minute volume and peak inspiration flow as well as inflammatory lesions in the lung morphology and effects on inflammatory markers (Sung *et al.,* 2008).

5.3 Human data

A topical wound dressing named Acticoat consists of a polyethylene mesh coated with nano-silver (average size 15 nm). There is one case report of silver poisoning after the use of Acticoat. On day 6 post injury, the patient developed a grayish discoloration, complained of being tired and having a lack of appetite. On day 7, silver levels in urine and blood were found to be elevated. After Acticoat was removed, the discoloration of the face gradually faded and liver function test returned to normal values (Trop *et al.,* 2006).

6. Cellular mechanisms of AgNPs-induced cytotoxicity

6.1 Generation of reactive oxygen species

ROS is the short-lived diffusible entities such as hydroxyl (·OH), peroxyl (ROO·) radicals and for some radical species of medium lifetime such as superoxide (O_2·) or nitroxyl radical (NO·). Due to its electron transport system (ETS), mitochondria are an important source of ROS within most mammalian cells. As shown in Figure 9, ETS normally produces 2-4% ROS from the electron flux. The increase of ROS level observed in pro-oxidant environments or the loss of redox equilibrium regulation is initially deactivated by ROS scavenger enzymes such as glutathione peroxidase, catalase and superoxide dismutase. However, excessive ROS production can lead to oxidative damage to mitochondrial proteins, membranes and DNA, impairing the ability of mitochondria to synthesize ATP and declination of mitochondrial membrane potential (MMP) which induces the opening of mitochondrial permeability transition pore (PTP). This oxidative stress condition results in irreversible permeability transition causing a release cytochrome c from mitochondria to cytoplasm and finally mitochondrial-regulated cell death occurs.

ROS generation and oxidative injury has been widely accepted as a vital mechanistic paradigm for AgNPs toxicity (Carlson *et al.,* 2008). Supporting this assumption, the increase of ROS levels accompanied by the decrease of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase as well as the increase of lipid peroxidation and DNA adducts formation have been detected after exposure to starch-coated and PVP-coated AgNPs (Arora *et al.,* 2008; Foldbjerg *et al.,* 2010). In additions, the correlation between ROS and indicators of cytotoxicity such as DNA damage, apoptosis and necrosis have been reported (Kim *et al.,* 2009). Interestingly, the cytotoxicity of PVP-coated AgNPs was greatly decreased by pretreatment with the ROS scavenger, N-acetyl-cysteine (Foldbjerg *et al.,* 2010).

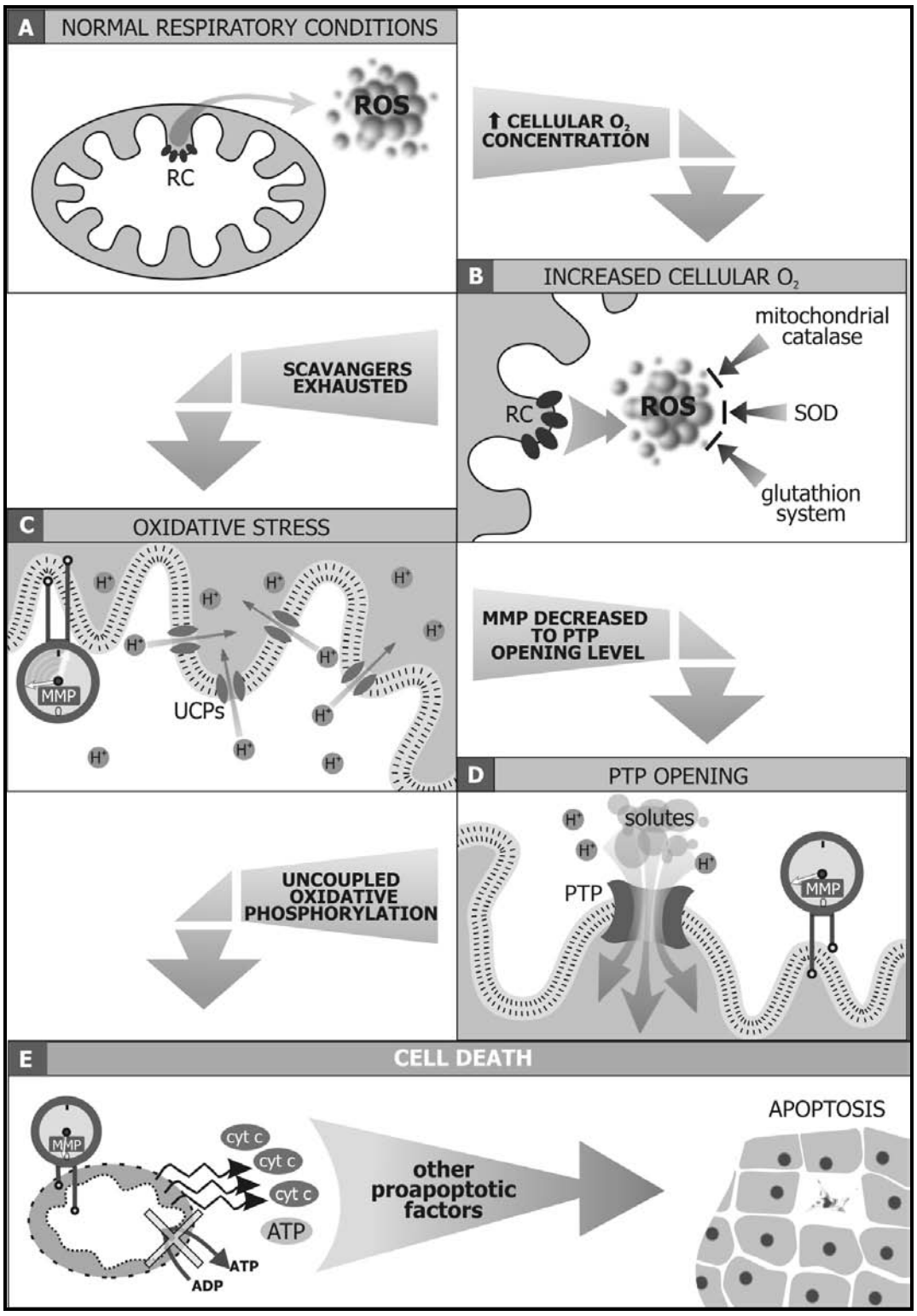


Figure 9 Mitochondrial defensive mechanisms against radical-induced

oxidative stress and the outcomes of excessive ROS production (Maiuri *et al.,* 2007)

6.2 Change of mitochondrial membrane potential

Mitochondrial membrane potential (MMP), the voltage gradient across the inner mitochondrial membrane, is generated from electron transfer in oxidative phosphorylation. Oxidative phosphorylation occurs by the close association of the electron carriers with protein molecules shown in Figure 10, These proteins guide the electrons along the respiratory chain so that the electrons move sequentially from one enzyme complex to another. Most importantly, the transfer of electrons is coupled to oriented proton (H^+) uptake and release. The net result is the pumping of proton (H^+) across from the matrix to the intermembrane space, driven by the energetically favorable flow of electrons, resulting in generation a voltage gradient with the inside negative and the outside positive called mitochondrial membrane potential (MMP).

Mitochondria are generally sensitive to many toxicant substances and closely related to oxidative balance in cells. As shown on Figure 9, impairment of mitochondrial function results in drastic consequences on redox potential equilibrium and normal cell function. Mitochondria are considered as major cellular compartments relevant for nanoparticles toxicity (Unfried *et al.,* 2007). As mentioned above, nanoparticles potentially damage mitochondrial structure through oxidative stress (Pan *et al.,* 2009; Sun *et al.,* 2011). As a result of structural damage, the loss of mitochondrial membrane integrity, opening of the permeability transition pore (PTP), ROS production and cell death may be occur (Li *et al.,* 2003; Xia *et al.,* 2007). Experimentally, particles initiate cytotoxicity by mitochondrial perturbation leading to increase of ROS production, decrease of mitochondrial membrane potential and induction of apoptosis (Upadhyay *et al.,* 2003). Furthermore, recent study has shown negatively dose-response in mitochondrial depolarization following exposure to metal oxide and silver nanoparticles (Hanna *et al.,* 2009; Hussain *et al.,* 2005). Presumably, NAC can prevent silica nanoparticles-induced mitochondrial depolarization (Xin *et al.,* 2010).

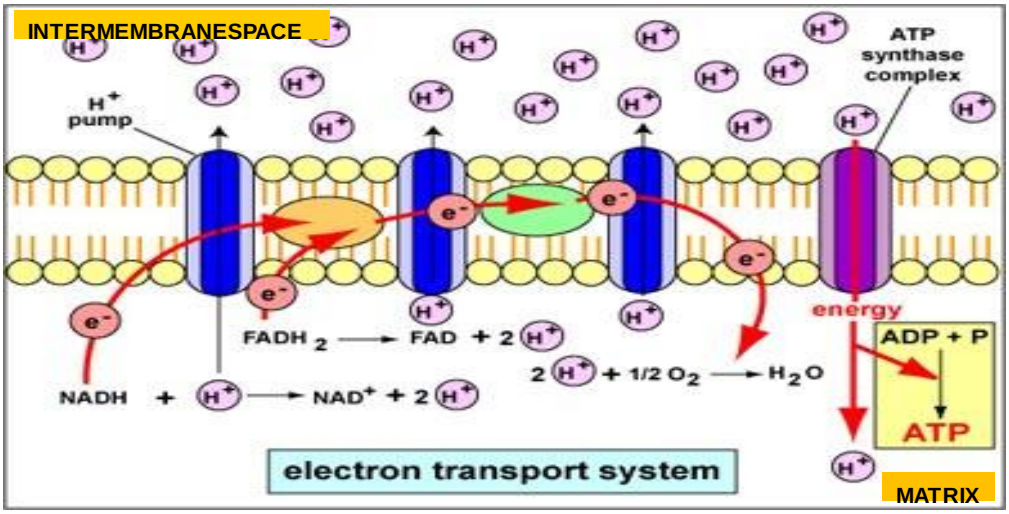


Figure 10 Electron transport system providing MMP (Lewis *et al.*, 2002)

6.3 Disturbance of normal cell cycle

Cell cycle is the complex process involved in the growth and proliferation of cells. As shown in Figure 11, four distinct phases could be recognized in cell cycle. G1 phase is required for cell growth and preparation of DNA synthesis. S phase is the DNA synthesis phase. G2 phase is needed for cell growth and preparation for mitosis and M-phase (mitosis) in which cells segregate duplicated chromosomes. Additionally, substances which cause the changes of cell cycle are probably assumed to interrupt normal process of cell progression. For examples, materials could damage DNA or disturb one of the vital steps of cell division such as S phase leading to disturbance of DNA synthesis and finally cell arrest or death.

Apart from cytotoxicity, disturbance of normal cell cycle is one of the effects that can be observed in AgNPs treated cells. Regarding the cell cycles, AgNPs induced G1 and S phase arrest (Park *et al.*, 2010). There have been reported that starch-coated silver NPs induced G2/M phase arrest and DNA damage in human glioblastoma cells and human fibroblasts (AshaRani *et al.*, 2009). Mainly compounds which contain anti-DNA synthesis effect can perturb S phase progression (Black *et*

al., 1989). However, little is known about effect of AgNPs on each phase of cell cycle.

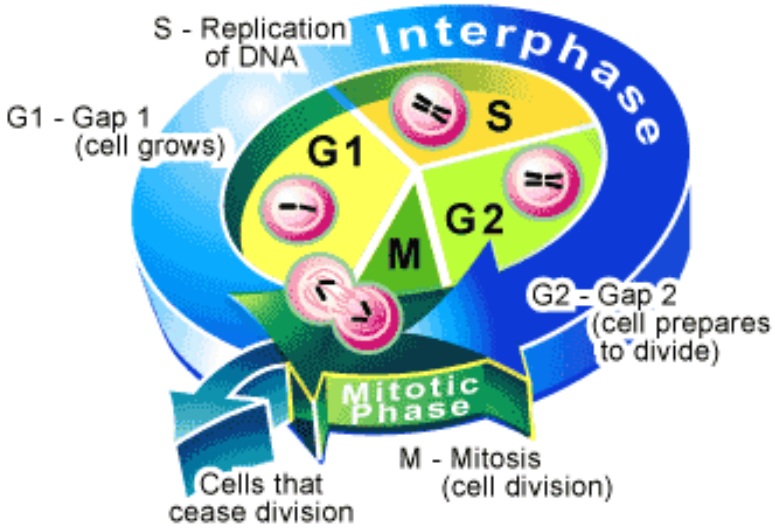


Figure 11 Each phase of cell cycle (Smith *et.al.,* 2001)

In additions, several studies have demonstrated apoptosis induced by NPs (Wang *et al.,* 2009; Ye *et al.,* 2010). Recent study has illustrated AgNPs-induced apoptosis in HeLa cells in a dose-dependent manner (Nobuhiko and Yasushi, 2009). Apoptosis defined as a programmed cell death is a process which can be induced by many toxic substances. During apoptosis, series of changes including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation occur. Consequently, large numbers of small fragments of DNA, whose sizes are about 180 base pairs, accumulate in the cell.

To analyze both effects of AgNPs, PI staining with flow cytometric analysis is the widely accepted method. The result from flow cytometric analysis provides both population of cells in each phase of cell cycle and apoptotic cells at once.

In detail, the G0/G1 phase is where cells spend most of their life. All cells except sex cells contain the same amount of DNA in the G0/G1phase. This is called the 2N amount of DNA. The phase of DNA synthesis called S-phase contains 4N of

DNA. Next stage of the cell cycle is called the G2/M which subsequently goes through mitosis. Thus, in G2/M phase, cell splits the 4N amount of DNA back into 2N and the cell returns to G1/G0. In the cell cycle analysis, a DNA staining dye such as PI, stoichiometrically binding to the DNA is used to yield the PI stained DNA. Then, this stained material is measured the emitted fluorescent signal by flow cytometer. These fluorescence data are considered as a cellular DNA content. According to the differences of DNA content in each phase of cell cycle, this method also enables the identification of cell distribution during various phases of cell cycle. Besides, this technique provides further information of DNA in cells. A presence of reduced fluorescent cells called subG1 peak which locates before G1. SubG1 peak indicates DNA break up into the small molecular weight fragments or occurrence of apoptosis (Figure 12) (Darzynkiewicz *et al.,* 1992).

In summary, the elevations of S or G2/M phase population and subG1 peak which are provided by flow cytometer as illustrated in Figure 12 indicate the change of cell cycle and the presence of apoptosis, respectively.

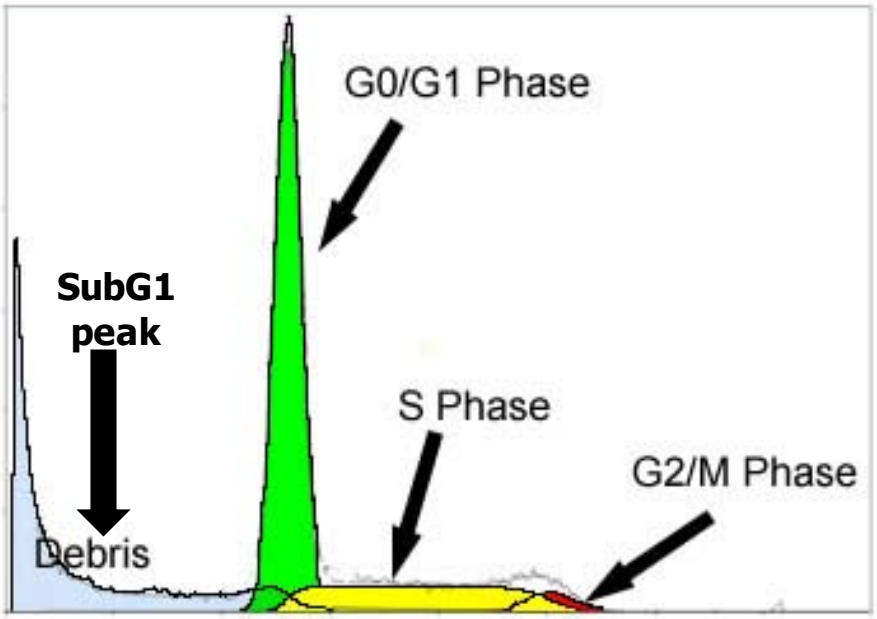


Figure 12 Histogram from flow cytometric analysis indicating subG1 peak and each phase of cell cycle (Mohamed *et al.*, 2001)

6.3.1 Proliferating cell nuclear antigen (PCNA)

A large number of proteins play key roles in each step of cell cycle. Among cell cycle-associated proteins, PCNA is the interesting protein containing versatile functions in various steps of cell cycle (Figure 13) (Maga and Hübscher, 2003). PCNA has been found in the nuclei of cells that undergo cell division. It is an evolutionarily well-conserved protein found in all eukaryotic species. It is synthesized in the early G1 and S phases of the cell cycle, is responsible for cell cycle regulation, DNA replication and DNA repair. PCNA was first shown to act as a processivity factor of DNA polymerase δ, which is required for DNA synthesis during replication. Furthermore, expression of the PCNA genes correlates with cell proliferation and DNA replication so it is assumed that PCNA can play a role in both processes (Strzalka and Ziemienowicz, 2010). PCNA exerts three functions such as a sliding clamp operator during DNA synthesis, a polymerase switch factor and a recruitment factor of DNA polymerase δ. PCNA down-regulation may indicate cell cycle arrest (Engel *et al.*, 2003). According to NIH established assays for proliferative effects of nanoparticles, immunostaining of PCNA correlates well with various methods (Jedd *et al.*, 2010). Therefore, analysis of PCNA expression could provide some mechanistic views of nanoparticles-induced cell cycle arrest.

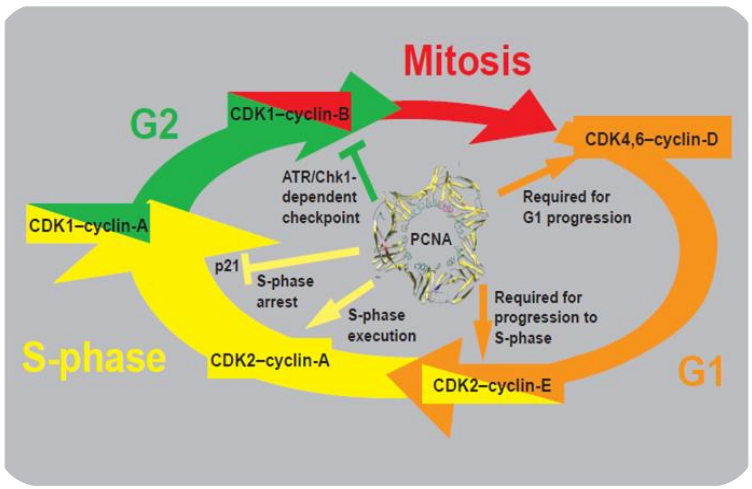


Figure 13 Roles of PCNA in regulation of cell cycle

(Maga and Hübscher, 2003)

CHAPTER III
MATERIALS AND METHODS

Materials

The human lung carcinoma epithelial cells (A549 or #CCL-185) cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Dulbecco's Phosphate-Buffered Saline (PBS), F-12K media and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY). The enhanced chemiluminescence system was purchased from GE Healthcare (Buckinghamshire, UK). AgNPs average size < 100 nm, in powder form, containing 99.5% metals basis were purchased from Sigma (St. Louis, MO), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-morpholino-sydnonimine (SIN-1), dimethyl sulfoxide (DMSO), N-acetyl cysteine (NAC), propidium iodide (PI), polyvinylidene fluoride (PVDF) membrane, Ribonuclease (RNase A), skimmed milk, and tetramethyl rhodamine ethyl ester (TMRE) were purchased from Sigma Chemical Company (St. Louis, MO). Primary antibodies including anti-PCNA, anti-GAPDH and goat anti-mouse IgG-HRP secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Instruments

1. 6-well plate (Corning, NY)
2. 10-mm dish (Corning, NY)
3. 96-well plate (Corning, NY)
4. Centrifuge (MX-305, TOMY, Japan)
5. Compact inverted microscope (CKX41, Olympus, Hamburg, Germany)
6. Elmasonic S30H sonicator (Elma, Germany)
7. Flow cytometer (BD FACSAriaTMII Cell Sorter, BD, NJ)
8. Gel Electrophoresis (MiniPROTEAN Tetra Cell, Biorad, CA)

9. Zetasizer Nanoseries S4700 (Malvern Instruments Ltd, Worcestershire, UK)
10. Microplate reader (Powerwave XS2, Biotek, VT)
11. Powerwave XS2 microplate reader (Biotek, VT)
12. Semidry transfer (TTRANS-BLOT SD, Biorad, CA)
13. SpectraMax M2 microplate fluorometer (Molecular Devices, Sunnyvale, CA)
14. Thermomixer (Thermomixer comfort, Eppendorf, Germany)
15. Transmission electron microscope (TEM; JEM-2010, Jeol)

Methods

1. Characterization of the AgNPs

The morphology of the AgNPs was visualized using a transmission electron microscopy (TEM). The hydrodynamic diameter and zeta potential of the particles were measured using a Zetasizer Nanoseries S4700. The 2 mg/ml stock AgNPs suspension was sonicated, diluted in deionized (DI) water to a final concentration of 200 μg/ml and measured immediately by dynamic light scattering (DLS).

2. Preparation of AgNP suspension for cell treatment

The stock AgNPs suspension (2 mg/ml) was prepared by dispersing AgNPs in deionized water and sonicating for 10 min. From this stock suspension, AgNP suspensions of various concentrations were prepared by serial dilution in the media.

3. Cell culture and treatment condition

A549 cells were maintained in F-12K media supplemented with 10% FBS in a 95% humidified atmosphere and 5% CO_2 at 37°C. After seeding cells for 24 h, all assays were conducted in complete F12-K media. The NAC (5 and/or 10 mM) pretreatment was done prior to AgNPs exposure for 1 h.

4. Morphological examination

A549 cells ($1.5x10^4$ cells/well) were seeded in 6-well plates and treated with 200 µg/ml of AgNPs for 24 h. Subsequently, cell morphology was visualized by a light microscope.

5. *In vitro* cytotoxicity assay

Cytotoxicity was assessed using MTT, a mitochondrial-based cell viability assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow, water soluble tetrazolium salt. MTT is converted to purple, water insoluble formazan on the reductive cleavage of its tetrazolium ring by mitochondrial reductase enzymes in living cells. Organic solvents such as, DMSO are used to solubilize this formazan. The solubilized formazan which can be detected by spectrophotometry is proportional to the number of living cells.

A549 cells ($5x10^3$ cells/well) were seeded in 96-well plates. After 24 h, the cell culture medium was discarded. The cells were treated with the media containing concentrations of AgNPs (25, 50, 100, 200 µg/ml) for 24 or 48 h. At the end of treatment, 25 µl of 5 mg/ml MTT solution (in PBS) was added to the cells and further incubated for 3 h. The medium was then discarded, and the resulted formazan crystal was dissolved with 100 µl of DMSO. Absorbance of the solution was measured at a wavelength of 570 nm using a microplate reader. Percentage of cell viability was calculated as the ratio of the mean of absorbance obtained for each condition to that of the control.

5.1 Effect of AgNPs on cell viability of A549 cells

To investigate the role of AgNPs on cell viability of A549 cells, cells were incubated in AgNPs at various concentrations (25, 50, 100, 200 µg/ml) for 24 and 48 h and cell viability was assessed by MTT assay.

5.2 Effect of antioxidant on cell viability of AgNPs treated cells

To examine whether the antioxidant, NAC could prevent AgNPs-related cytotoxicity, cells were incubated with NAC (5 and 10 mM) 1 h prior to AgNPs exposure. Then, cells were incubated with AgNPs at various concentrations (25, 50, 100, 200 μg/ml) for 24 and 48 h and cell viability was determined by MTT assay.

6. Intracellular reactive oxygen species assay: DCF assay

Intracellular reactive oxygen species after AgNPs treatment was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA), a dye which can permeate membranes where it is deesterified to be non-fluorescent DCFH by esterase enzymes. Intracellular ROS reacting with non-fluorescent DCFH, the final reaction yields fluorescent compound dichlorofluorescein (DCF) which can be detected by spectrophotometer.

According to this principle, A549 cells ($5x10^3$ cells/well) were seeded in black 96-well plates. The cells were incubated with 50 μM DCFH-DA for 40 min followed by exposure to AgNPs for 3 h. The fluorescent intensity was detected using a microplate fluorometer at excitation and emission wavelength of 485 nm and 528 nm, respectively. SIN-1 was used as a positive control.

6.1 Effect of AgNPs on intracellular ROS generation in A549 cells

To investigate the role of AgNPs on intracellular ROS formation, cells were incubated in AgNPs at various concentrations (25, 50, 100, 200 µg/ml) or SIN-1 (20 and 40 µM) for 3 h. Then, intracellular ROS was assessed by DCF assay.

6.2 Effect of antioxidant on AgNPs induced ROS generation in A549 cells

To evaluate whether the antioxidant, NAC could prevent AgNPs-induced ROS formation, cells were incubated with NAC (5 and 10 mM) 1 h prior to AgNPs exposure. Then, cells were incubated with AgNPs at various concentrations (25, 50, 100, 200 µg/ml) or SIN-1 (40 µM) for 3 h. Then, the intracellular ROS was assessed by DCF assay.

7. Mitochondrial membrane potential (MMP) analysis

Tetramethylrhodamine ethylester (TMRE) assay was conducted to analyze MMP following AgNPs exposure. TMRE, a fluorescent permeable cationic lipophilic dye, is used as a fluorescent probe to monitor the membrane potential of mitochondria. Depending on its charge, TMRE has a tendency to accumulate in the matrix which contains negative charge resulting from equilibrium of electron transport system. Thus, this fluorescent intensity detected by flow cytometer designates the normal level of MMP in active mitochondria.

According to this method, TMRE stock was prepared at a concentration of 25 mM in DMSO and stored at -20°C. A549 cells ($1.5x10^5$ cells/well) were seeded in 6-well plates. Then, cells were incubated in media containing 0, 100 and 200 µg/ml of AgNPs for 24, 48 and 72 h. At the end of treatment, the media was removed. Cells were washed with PBS, trypsinized and harvested in the media. The cell suspension was centrifuged and washed twice with PBS. The cell pellets were incubated with 25 µM TMRE (in PBS) at 37^oC for 15 min in dark conditions. Then, the stained cells were washed twice with PBS. All samples were then analyzed by a flow cytometer.

The data from the histograms were analyzed using BDFACSDiva software. Finally, the percentage of TMRE stained cells and total cells was calculated.

7.1 Effect of AgNPs on mitochondrial membrane potential in A549 cells

To examine the role of AgNPs on MMP of A549 cells, cells were incubated with AgNPs at high concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and MMP was analyzed by TMRE staining with Fluorescence Activated Cell Sorting (FACS) analysis.

7.2 Effect of the antioxidant on AgNPs-perturbed mitochondrial membrane potential in A549 cells

To evaluate whether the antioxidant, NAC could prevent AgNPs-perturbed mitochondrial membrane potential, cells were incubated with 10 mM NAC 1 h prior to AgNPs exposure. Then, cells were incubated in AgNPs at high concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and MMP was analyzed by TMRE staining with FACS analysis.

8. Cell cycle analysis

The nuclear DNA content can be quantitatively measured by DNA staining dyes and flow cytometry. Initially, a DNA staining dye such as propidium iodide (PI), which binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells. Amount of incorporated dye is proportional to the amount of DNA. The stained material is then measured by flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescent emission from the cell. Thereafter, such fluorescent data are considered a measurement of the cellular DNA content. According to the difference of DNA content in each phase of cell cycle, flow cytometry also enables the identification of

cell distribution during various phases of cell cycle. Four distinct phases could be recognized in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). However, G2- and M-phase, which both have an identical DNA content could not be discriminated based on their differences in DNA content. In this experiment, cell cycle analysis was performed by staining the DNA with PI followed by flow cytometric analysis. This method provides the DNA histogram giving a static picture of the proportion of cells in different phases of the cell cycle.

A549 cells ($1.5x10^4$ cells/well) were seeded in 6-well plates and incubated for 24 h. The media were removed and the cells were cultured in the media containing 0, 100 and 200 μg/ml of AgNPs for 24, 48 and 72 h. At the end of treatment, the cells were trypsinized and harvested in the medium. The cells were centrifuged, washed twice with PBS and fixed with 80% ethanol. The cells were kept at -20°C. Before analysis, cells were centrifuged, washed with PBS and stained with 50 μg/ml PI in 200 μg/ml RNase A and incubated at 37 °C for 30 min and at 4 °C until analysis. The FACS analysis of cell cycle was performed using a flow cytometer. The data from histograms were analyzes using BDFACSDiva software. The percentage of cells in each phase of the cell cycle was compared with those of the controls.

8.1 Effect of AgNPs on cell cycle of A549 cells

To examine the role of AgNPs on cell cycle of A549 cells, cells were incubated with AgNPs at high concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and cell cycle was analyzed by PI staining with FACS analysis.

8.2 Effect of antioxidant on the change of cell cycle generated by AgNPs in A549 cells

To evaluate whether the antioxidant, NAC could modulate the change of cell cycle caused by AgNPs, cells were incubated with 10 mM NAC 1 h prior to AgNPs exposure. Then, cells were incubated in AgNPs at concentrations of 100 and 200

μg/ml for 24, 48 and 72 h. Cell cycle of A549 cells was analyzed by PI staining followed by FACS analysis.

9. Proliferating Cell Nuclear Antigen (PCNA) expression analysis

To provide mechanistic view of the change of cell cycle induced by AgNPs, cell cycle-associated proteins should be assessed. Among these, PCNA, a protein synthesized in the early G1 and S phases of the cell cycle, is responsible for cell cycle regulation, DNA replication and DNA repair (Strzalka and Ziemienowicz, 2010). NIH guideline for investigating proliferative effects of nanoparticles, stated that immunostaining of PCNA correlates well with other cell proliferation detection methods (Jedd *et al.,* 2010). Thus, change of PCNA protein expression after exposure to AgNPs was investigated by Western blot analysis.

A549 cells ($8.5x10^5$ cells) were seeded in 10-mm dishes and incubated for 24 h. Then, the cells were incubated in media containing 0, 100 and 200 μg/ml of AgNPs for 48 and 72 h. At the end of treatment, the cellular protein was extracted and subjected to Western blot analysis. Briefly, proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The membrane was blocked with 5% skimmed milk in PBS overnight. Then, the membranes were incubated with anti-PCNA (1:100 dilution) for 2 h at room temperature. After washed with PBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution) for 30 min at room temperature. Finally, the amount of PCNA was quantified by the enhanced chemiluminescence system. GAPDH, an internal standard, was detected by anti-GAPDH (1:100 dilution). Three independent experiments were performed. Band intensity was analyzed by Scion Image (4.0) software.

9.1 Effect of AgNPs on PCNA protein expression in A549 cells

To investigate the role of AgNPs on PCNA protein expression with A549 cells, cells were incubated with AgNPs at high concentrations (100 and 200 μg/ml) for 48 and 72 h and the change of PCNA protein expression after exposed to AgNPs was detected by Western blot analysis.

9.2 Effect of antioxidant on the change of PCNA protein expression modulated by AgNPs in A549 cells

To evaluate whether the antioxidant, NAC could prevent the change of PCNA protein expression modulated by AgNPs, cells were incubated with 10 mM NAC 1 h prior to AgNPs exposure. Then, cells were incubated with AgNPs at different concentrations (100 and 200 μg/ml) for 48 and 72 h and the change of PCNA protein expression was detected by Western blot analysis.

10. Statistical analysis

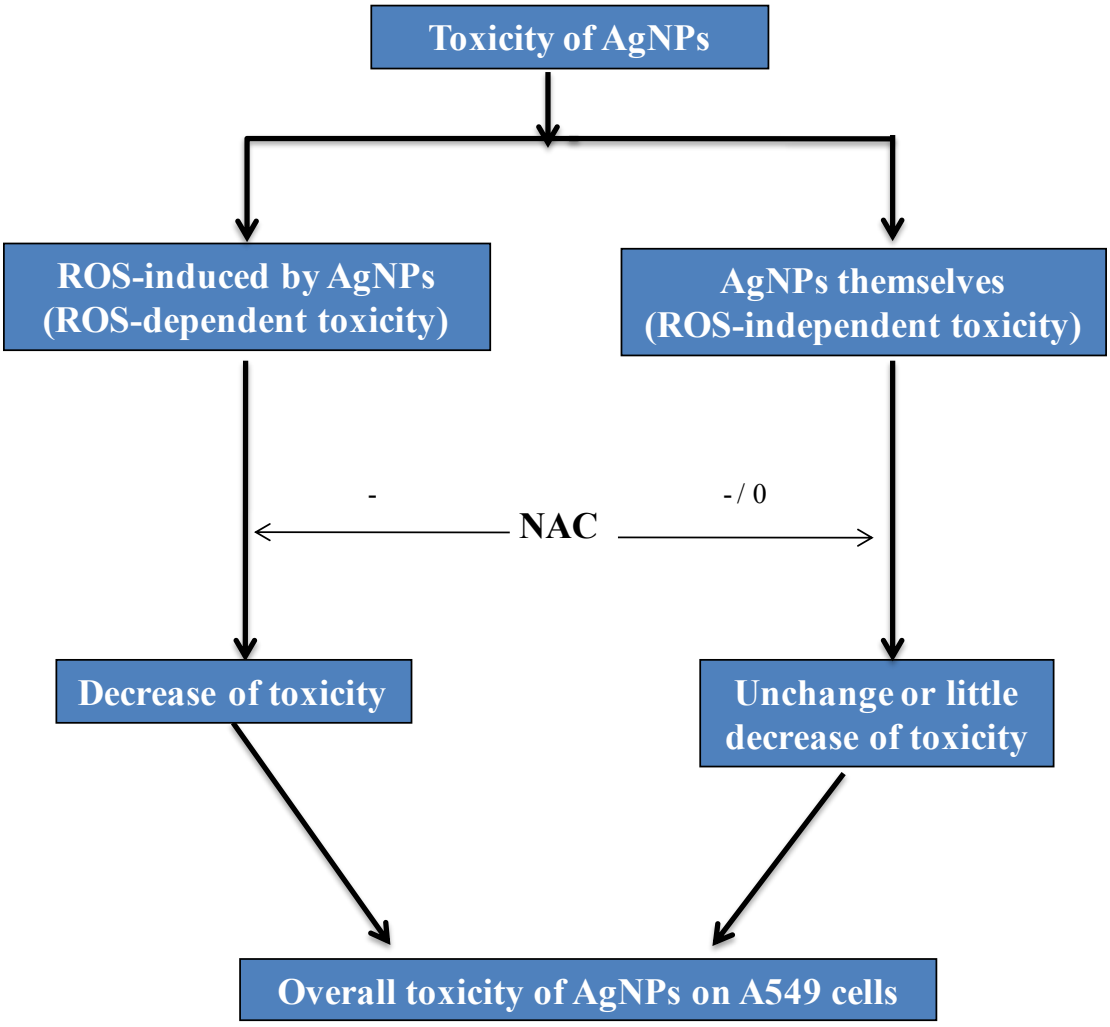
All data were presented as means ± standard deviation (S.D.). Three independent experiments were conducted to confirm the reproducibility of the sets of experiments. Data shown in the figures were a representative set of experiments. Difference among groups was determined by one way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. The level of statistical significance was set at p value < 0.05. All statistic data were analyzed by SPSS program version 16.0 (Network license purchased by Chulalongkorn university)

11. Experimental Design and the conceptual framework

This study was divided into 7 parts as follows:

1. Characterization of silver nanoparticles (using DLS and TEM)
2. Determination morphological effect of AgNPs on A549 cells (using light microscope)
3. Determination cytotoxicity effect of AgNPs on A549 cells (using MTT assay)
4. Determination effect of AgNPs on intracellular ROS generation in A549 cells (using DCF assay)
5. Determination effect of AgNPs on mitochondria membrane potential in A549 cells (using TMRE assay)
6. Determination effect of AgNPs on cell cycle in A549 cells (using PI staining assay and FACS analysis)
7. Determination effect of AgNPs on PCNA protein expression in A549 cells (using Western blot analysis)

Conceptual framework



CHAPTER IV
RESULTS

1. Characterization of the AgNPs

The earliest step of the nanoparticles-associated toxicological study is characterization of nanoparticles. The morphology and average size of AgNPs was determined by TEM. As shown in Figure 14, most of the AgNPs were spherical and approximately 40-50 nm in diameter in aqueous solution. However, agglomerates of AgNPs, ranging in size from 100 to 300 nm, were observed after incubation in cell-free culture media. The hydrodynamic radius in culture medium at 24 h detected by DLS method was summarized in Table 4. The hydrodynamic size of AgNPs in media after sonication for 0, 1, 5, 10 min was 750.03 ± 223.15 nm, 185.58 ± 27.40 nm, 190.41 ± 22.86 and 182.65 ± 12.97 nm, respectively. Besides, the absolute figure of zeta potential of all samples were above 30 indicating the stability of AgNPs in this media.

2. Morphological examination

Microscopic observation showed no massive cell death in AgNPs treated cells (Figure 15). However, treated cells showed distinct morphological changes such as a few cellular extensions. These irregular shapes pinpointed that AgNPs could potentially disturb cytoskeletal functions.

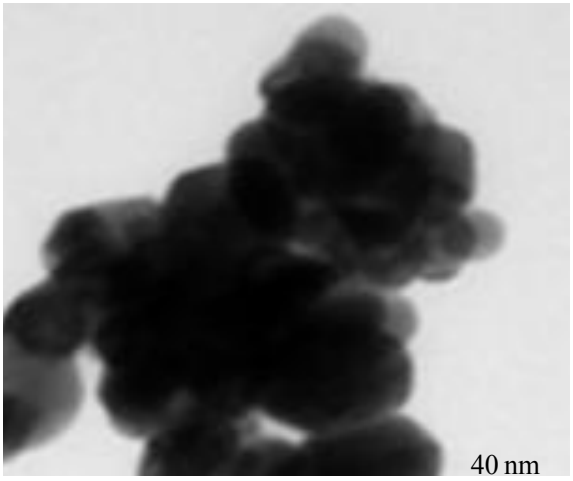
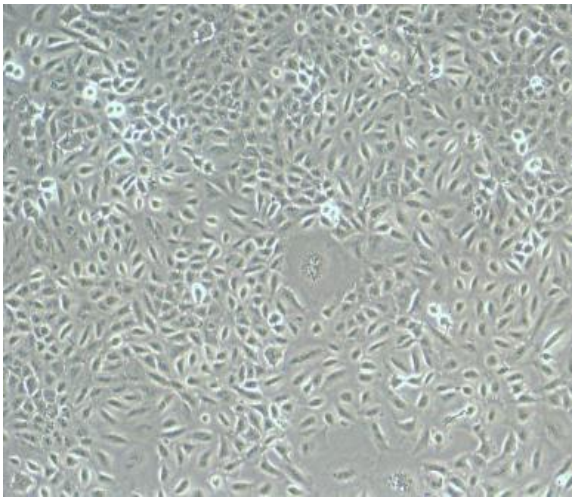


Figure 14 TEM image of silver nanoparticles

Table 4 Characterization of AgNPs

Sonication time (min)	Mean diameter (nm)	Zeta poential
0	750.03 223.15	-30.53 1.74
1	185.58 27.40	-31.32 1.33
5	190.41 22.86	-33.39 2.11
10	182.65 12.97	-34.14 3.59

(A)



(B)

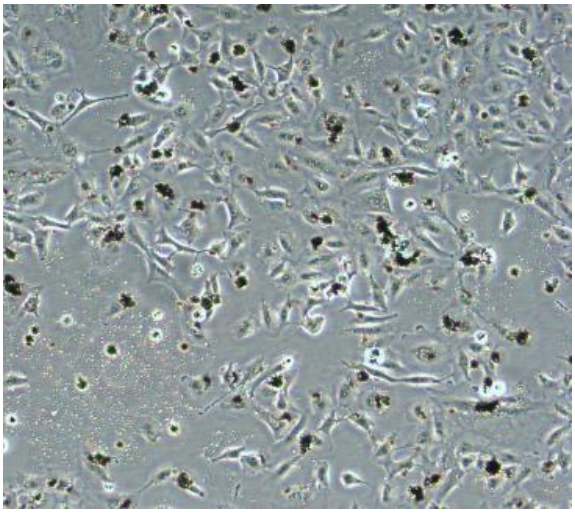


Figure 15 Microscopic examination of A549 cells without nanoparticle treatment (A) and with 200 μg/ml of AgNPs for 24 h (B).

3. *In vitro* cytotoxicity assay

Cytotoxicity was assessed using MTT, a mitochondrial-based cell viability assay. MTT is converted to purple, water insoluble formazan and DMSO is used to generate solubilized formazan. Its intensity detected by spectrophotometry is proportional to the number of living cells.

3.1 Effect of AgNPs on cell viability of A549 cells

To investigate the role of AgNPs on viability of A549 cells, cells were incubated in AgNPs at various concentrations for 24 and 48 h and cell viability was assessed by MTT assay. As shown in Figure 16 and 17, low concentration of AgNPs (25 μg/ml) did not cause significant ($P > 0.05$) toxicity on A549 cells either at 24 or 48 h of AgNPs incubation. Higher concentrations of AgNPs (50, 100 and 200 μg/ml) caused significant ($P < 0.05$) decrease of cell viability in a concentration-dependent manner as compared to their corresponding control either at 24 or 48 h of AgNPs incubation.

3.2 Effect of antioxidant on cell viability of AgNPs treated cells

To examine whether antioxidant, NAC could prevent AgNPs-related cytotoxicity, cells were incubated with NAC (5 and 10 mM) 1 h prior to AgNPs exposure. Then, cells were incubated in AgNPs at various concentrations for 24 and 48 h and cell viability was determined by MTT assay. Cell viability in NAC pretreatment groups was significantly increased as compared to the corresponding non-pretreatment groups. The addition of NAC clearly protected A549 cells from AgNPs induced cell death. In all 24 h-treated groups (Figure 16), viability of cells pretreated with 10 mM NAC was significantly ($P < 0.05$) different from the corresponding untreated controls while those with 5 mM NAC pretreatment were not. However, at 48 h of AgNPs incubation, both 5 and 10 mM NAC pretreatment significantly ($P < 0.05$) improved cell viability in a concentration-dependent manner

(Figure 17). Among the cells treated with the same concentration of AgNPs, higher cell viability was shown in groups which were pretreated with the higher concentration of NAC. This result indicated that AgNPs affected cell viability and pretreatment cells with NAC could attenuate AgNPs toxicity. Both effect showed in a concentration-dependent manner.

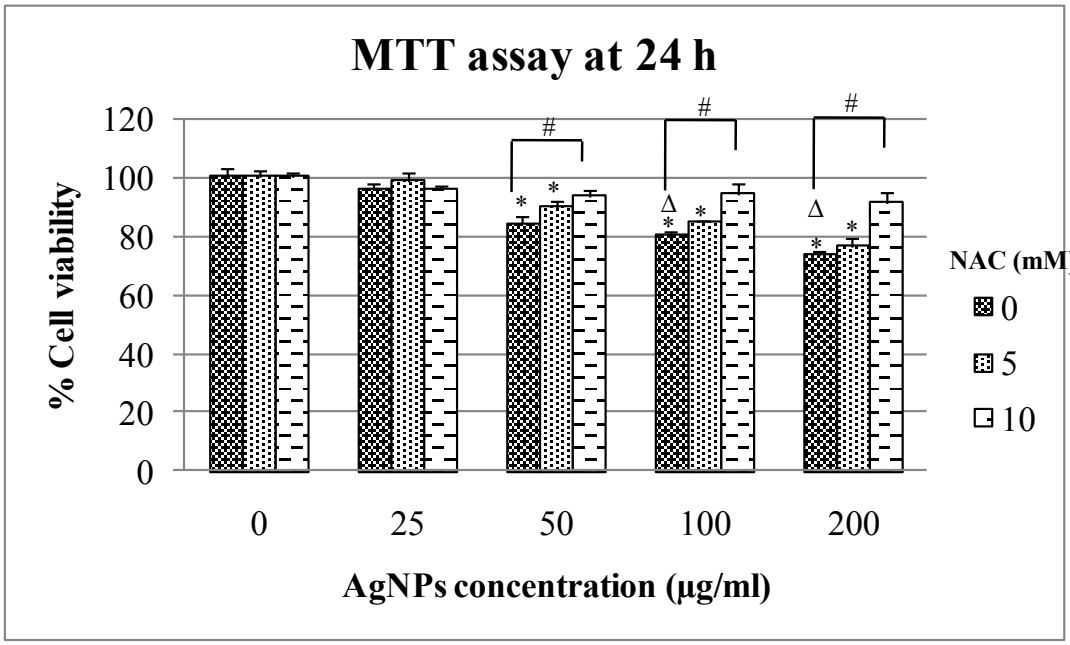


Figure 16 Effect of AgNPs on cell viability and effect of NAC on cell viability of AgNPs treated cells. Cells were exposed to different concentrations of AgNPs with or without pretreatment with NAC (5 or 10 mM) 1 h prior to AgNPs exposure for 24 h. Cell viability was assessed by MTT assay

The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC pretreatment group vs non-NAC pretreatment control group at the same concentration of AgNPs

Δ $P < 0.05$; 100 and 200 µg/ml of AgNPs vs 25 µg/ml of AgNPs without pretreatment of NAC

$P < 0.05$; 200 µg/ml of AgNPs vs 50 µg/ml of AgNPs without pretreatment of NAC

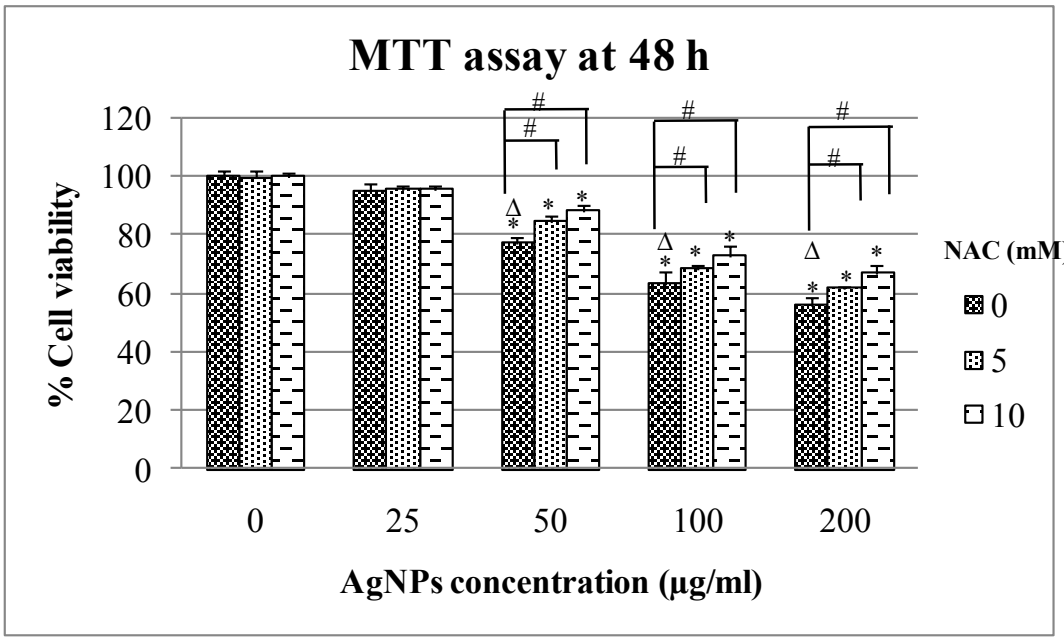


Figure 17 Effect of AgNPs on cell viability and effect of NAC on cell viability of AgNPs treated cells. Cells were exposed to different concentrations of AgNPs with or without pretreatment with NAC (5 or 10 mM) 1 h prior to AgNPs exposure for 48 h. Cell viability was assessed by MTT assay

The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC pretreatment group vs non-NAC pretreatment control group at the same concentration of AgNPs

Δ $P < 0.05$; 50, 100 and 200 µg/ml of AgNPs vs 25 µg/ml of AgNPs without pretreatment of NAC

$P < 0.05$; 200 µg/ml of AgNPs vs 50 µg/ml of AgNPs without pretreatment of NAC

4. Intracellular reactive oxygen species assay: DCF assay

Intracellular reactive oxygen species after AgNPs treatment was measured using DCFH-DA. The final reaction yields a fluorescent compound, DCF of which the fluorescence intensity represents the oxidative stress condition in cells.

4.1 Effect of AgNPs on intracellular ROS generation in A549 cells

To investigate the role of AgNPs on intracellular ROS formation, cells were incubated in AgNPs at various concentrations (25, 50, 100, 200 μg/ml) and SIN-1 (40 μM) for 3 h Then, the intracellular ROS was assessed by DCF assay. As shown in Figure 18, low concentrations of AgNPs (25, 50 μg/ml) did not generate significant ($P > 0.05$) increase in intracellular ROS. Nevertheless, both high concentrations of AgNPs (100 and 200 μg/ml) generated significant ($P < 0.05$) increase in intracellular ROS in a concentration-dependent manner. In accordance, SIN-1, the ROS generating agent, showed an increase of intracellular ROS with approximately 5.34 ± 0.12 fold of the control.

4.2 Effect of antioxidant on AgNPs induced ROS generation in A549 cells

To evaluate whether NAC could prevent the AgNPs-induced ROS formation, cells were incubated with NAC (5 and 10 mM) 1 h prior to AgNPs exposure. Then, cells were incubated with AgNPs (25, 50, 100, 200 μg/ml) for 3 h or SIN-1 (40 μM) for 3 h. Then, the intracellular ROS was assessed by DCF assay. The result showed that NAC pretreatment significantly decreased both AgNPs and SIN-1 induced ROS generation as compared to the corresponding non-pretreatment groups. At 100 μg/ml of AgNPs, both 5 and 10 mM of NAC could significantly decrease ROS formation. However, at 200 μg/ml of AgNPs, only 10 mM NAC could be able to decrease ROS formation significantly. For SIN-1, both 5 and 10 mM of NAC significantly attenuate SIN-1 induced ROS formation in A549 cells (Figure 18).

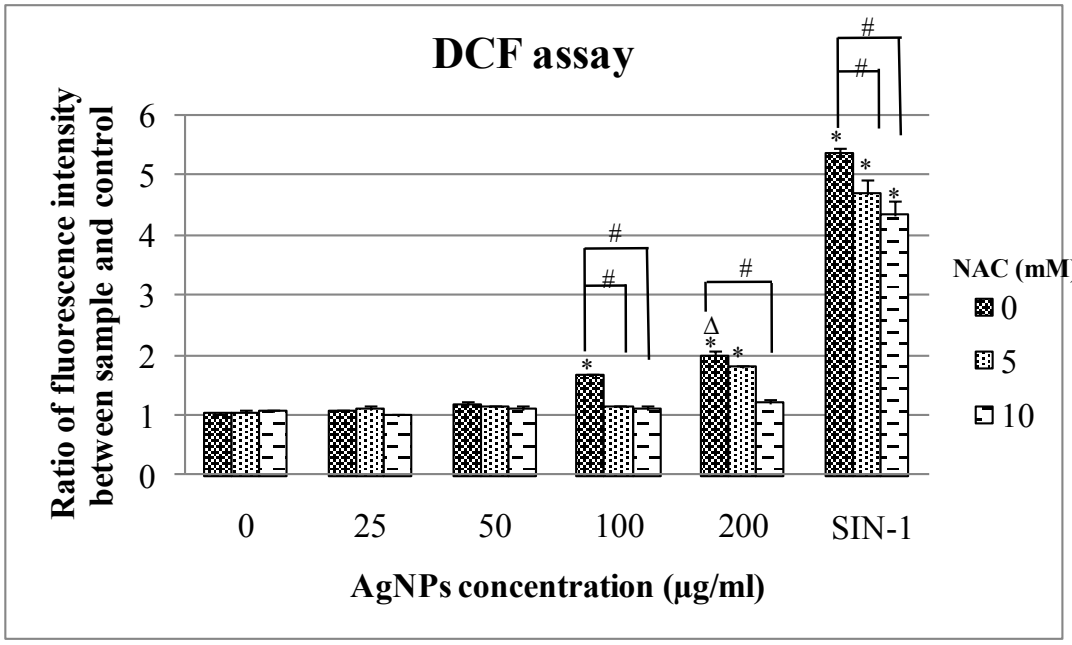


Figure 18 Effect of AgNPs on intracellular ROS and effect of NAC on ROS formation of AgNPs treated cells. Cells were pretreated with NAC (5 or 10 mM) for 1 h and exposed to different concentrations of AgNPs for 3 h. ROS level was measured by the fluorescence of DCF. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC pretreatment group vs non-NAC pretreatment control group at the same concentration of AgNPs or SIN-1

Δ $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs without pretreatment of NAC

5. Mitochondrial membrane potential analysis

TMRE assay was conducted to analyze MMP following AgNPs exposure.

5.1 Effect of AgNPs on mitochondrial membrane potential in A549 cells

To examine the role of AgNPs on MMP of A549 cells, cells were incubated in AgNPs at high concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and MMP was analyzed by TMRE staining with FACS analysis. As illustrated in Figure 19, the percentage of fluorescent cells significantly ($P < 0.05$) decreased in both AgNPs (100 and 200 μg/ml) exposure groups for 24, 48 and 72 h in a concentration-dependent manner.

5.2 Effect of antioxidant on AgNPs-perturbed mitochondrial membrane potential in A549 cells

To evaluate whether NAC could prevent AgNPs-perturbed mitochondrial membrane potential, cells were incubated with 10 mM NAC 1 h prior to AgNPs exposure. Then, cells were incubated in AgNPs at high concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and MMP was analyzed by TMRE staining with FACS analysis. As shown in Figure 20-22, the percentage of fluorescent cells significantly ($P < 0.05$) increased when cells were exposed to 100 μg/ml of AgNPs for 24, 48 and 72 h indicating that NAC could possibly attenuate the effect of AgNPs on MMP. However, NAC at 10 mM could not be able to attenuate the MMP declination induced by AgNPs at the higher concentration of 200 μg/ml.

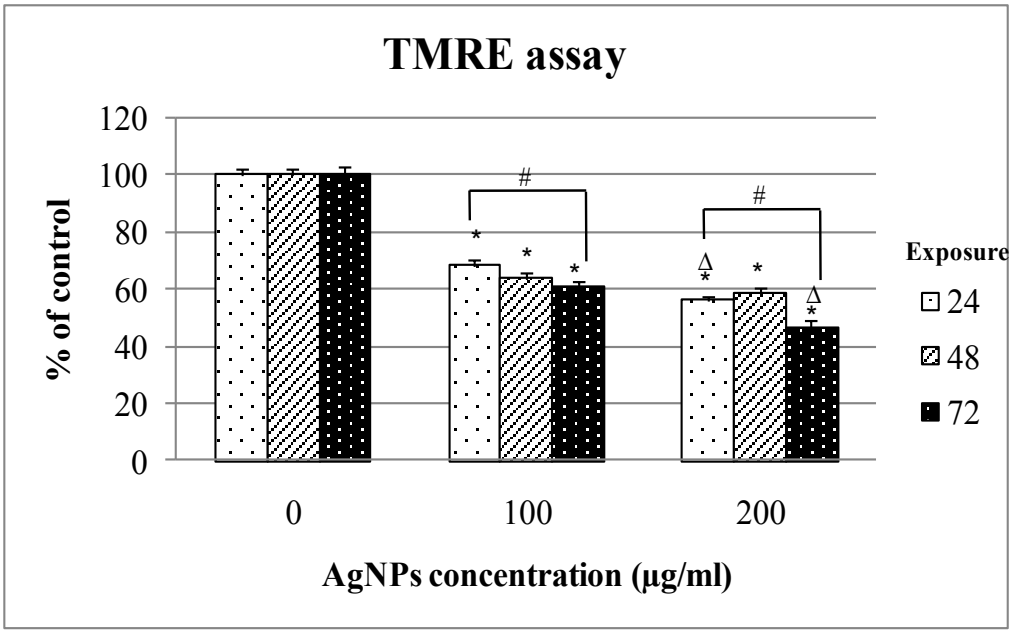


Figure 19 Effect of AgNPs on mitochondrial membrane potential in A549 cells. Cells were incubated with different concentrations of AgNPs for 24, 48 and 72 h. MMP was measured by the fluorescence of TMRE. The data shown are mean ± SD of % of fluorescent cells as compared to the controls of three independent experiments.

* $P < 0.05$; AgNPs treated group vs Non-AgNPs treated group at the same duration of incubation.

# $P < 0.05$; 24 h exposure groups vs 72 h exposure groups at the same concentration of AgNPs

Δ $P < 0.05$; 200 µg/ml of AgNPs vs 100 µg/ml of AgNPs at the same exposure time

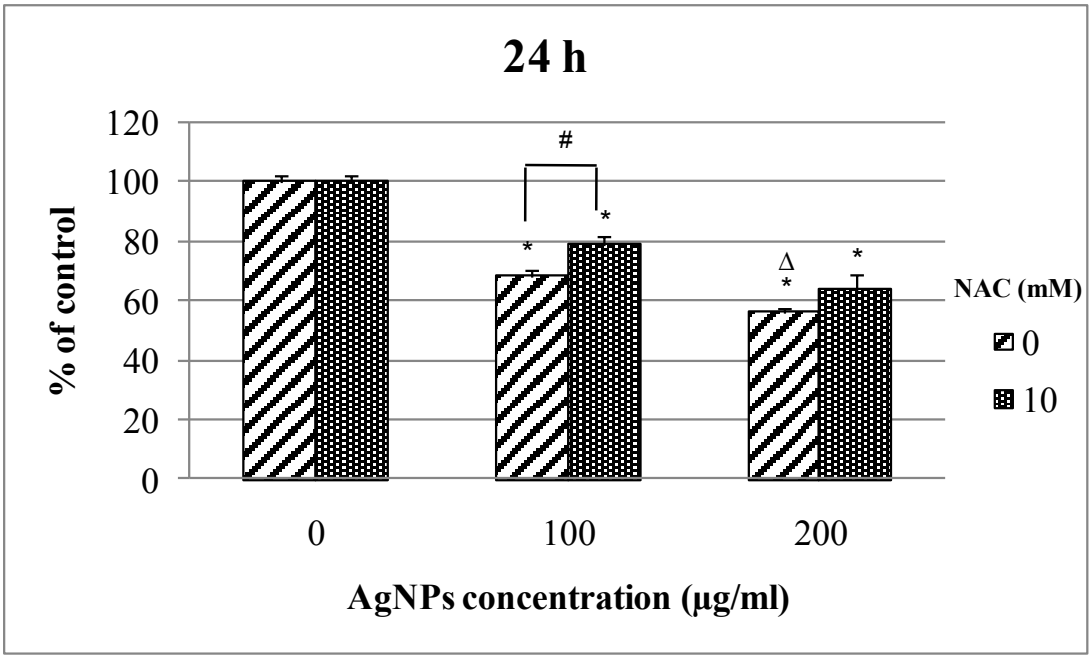


Figure 20 Effect of NAC on AgNPs-perturbed mitochondrial membrane potential in A549 cells. Cells were pretreated with NAC (10 mM) for 1 h and exposed to different concentrations of AgNPs for 24 h. MMP was measured by the fluorescence of TMRE. The data shown are mean ± SD of % of fluorescent cells as compared to the controls of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

Δ $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs without pretreatment of NAC

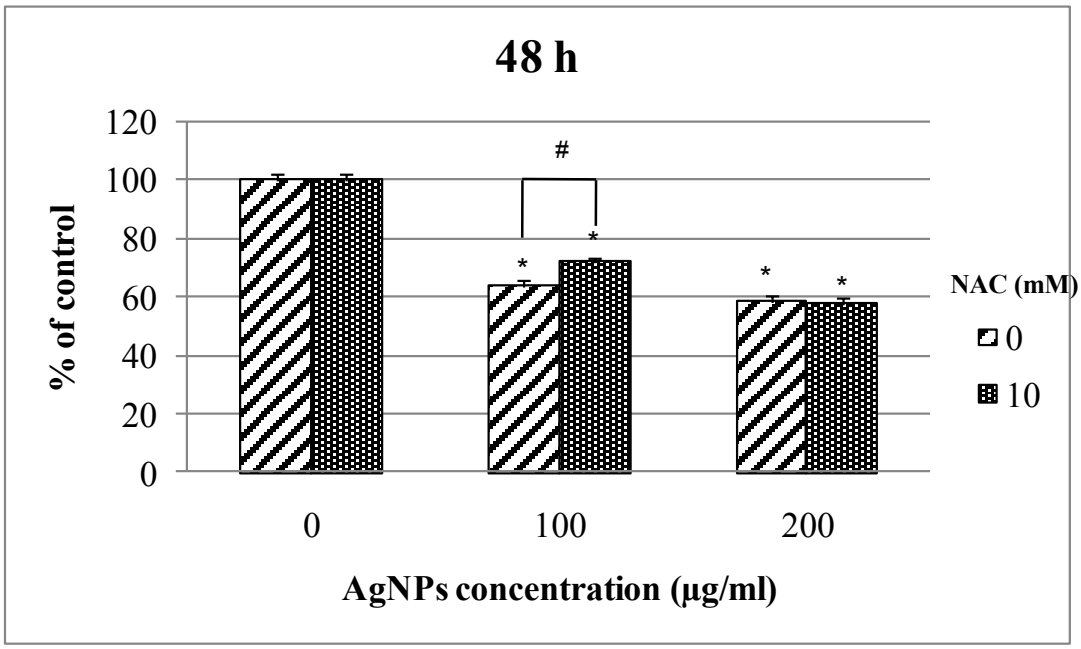


Figure 21 Effect of NAC on AgNPs-perturbed mitochondrial membrane potential in A549 cells. Cells were pretreated with NAC (10 mM) for 1 h and exposed to different concentrations of AgNPs for 48 h. MMP was measured by the fluorescence of TMRE. The data shown are mean ± SD of % of fluorescent cells as compared to the controls of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

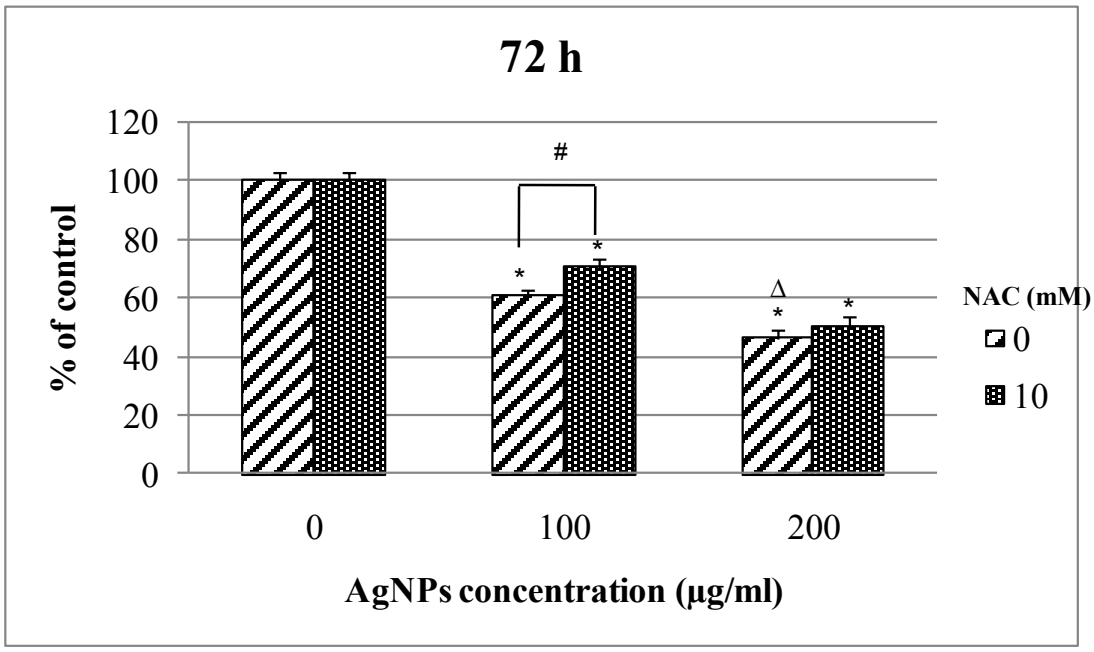


Figure 22 Effect of NAC on AgNPs-perturbed mitochondrial membrane potential in A549 cells. Cells were pretreated with NAC (10 mM) for 1 h and exposed to different concentrations of AgNPs for 72 h. MMP was measured by the fluorescence of TMRE. The data shown are mean ± SD of % of fluorescent cells as compared to the controls of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

Δ $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs without pretreatment of NAC

6. Cell cycle analysis

Cell cycle analysis was performed by staining DNA with PI followed by flow cytometric analysis. This method provides the DNA histogram giving the population of cells in different phases of the cell cycle. By this method, the accumulation of cells in subG1, gap1 (G1), DNA synthesis (S) and in gap2/mitosis (G2/M) phase will be detected.

6.1 Effect of AgNPs on cell cycle of A549 cells

To examine effect of AgNPs on cell cycle of A549 cells, cells were incubated with AgNPs at high concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and cell cycle was analyzed by PI staining with FACS analysis. The percentage of apoptotic cells, as detected in a sub-G1 peak, significantly increased in a concentration dependent manner after treatment with AgNPs (Figure 23).

As shown in Figure 24, AgNPs caused a significant increase of cells in the S phase varied from 15-20% and a corresponding decrease in G1 population. The percentage of cells in G2/M phase was less affected. This result demonstrated that AgNPs could possibly affect proliferation of A549 cells via induction of S phase arrest.

6.2 Effect of antioxidant on the change of cell cycle generated by AgNPs in A549 cells

To evaluate whether NAC could modulate the change of cell cycle generated by AgNPs, cells were incubated with 10 mM NAC 1 h prior to AgNPs exposure. Then, cells were incubated with AgNPs at different concentrations (100 and 200 μg/ml) for 24, 48 and 72 h and cell cycle was analyzed by PI staining with FACS analysis. According to Figure 25-27, pretreatment of NAC significantly ($P < 0.05$) decreased subG1 population indicating that NAC could prevent AgNPs-induced apoptosis. However, no statistical significance was observed between S phase

population of NAC-pretreated groups and non-NAC pretreated controls (Figure 29, 30) when cells were treated with AgNPs at 100 and 200 µg/ml of both 48 and 72 h of incubation. However, at 24 h of AgNPs exposure, NAC pretreatment attenuated an increase S phase population only at 200 µg/ml of AgNPs (Figure 28).

Taken together, AgNPs induced apoptosis (subG1) could be prevented by NAC pretreatment while AgNPs induced S-phase arrest could not be prevented by NAC pretreatment.

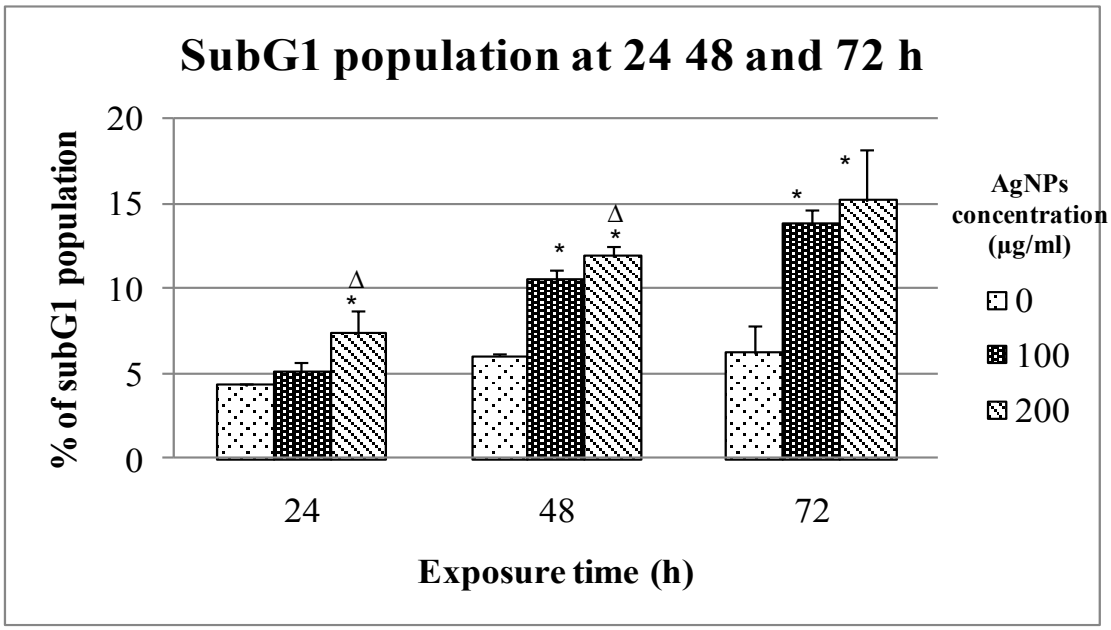


Figure 23 Effect of AgNPs on cell cycle of A549 cells. The percent of cells (events) under each area was generated. The data shown are mean ± SD of % of subG1 population as compared to the controls of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the AgNPs untreated group

Δ $P < 0.05$; 200 µg/ml of AgNPs vs 100 µg/ml of AgNPs of the same exposure time

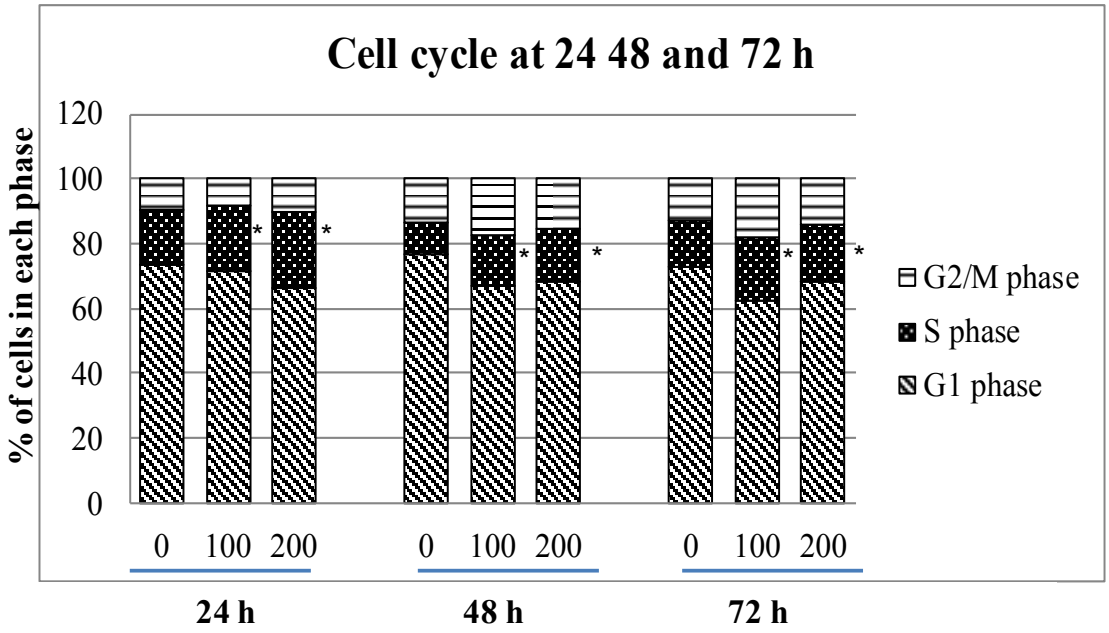


Figure 24 Effect of AgNPs on cell cycle of A549 cells. The percent of cells (events) under each area was generated and the data were statistical analysis by SPSS (16.0) software.

* $P < 0.05$; AgNPs treated group vs the AgNPs untreated group

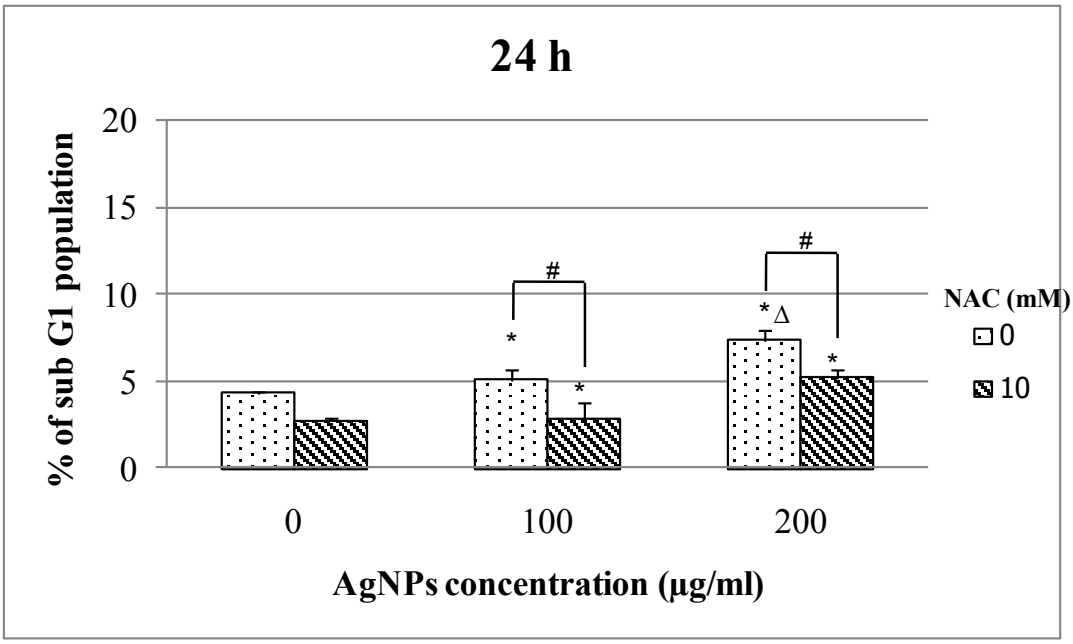


Figure 25 Effect of NAC on the change of subG1 population caused by AgNPs in A549 cells. Cells were treated with 10 mM NAC for 1 h and exposed to different concentrations (100, 200 μg/ml) of AgNPs for 24 h. Data shown are percentage of cells in subG1. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

Δ $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs without pretreatment of NAC

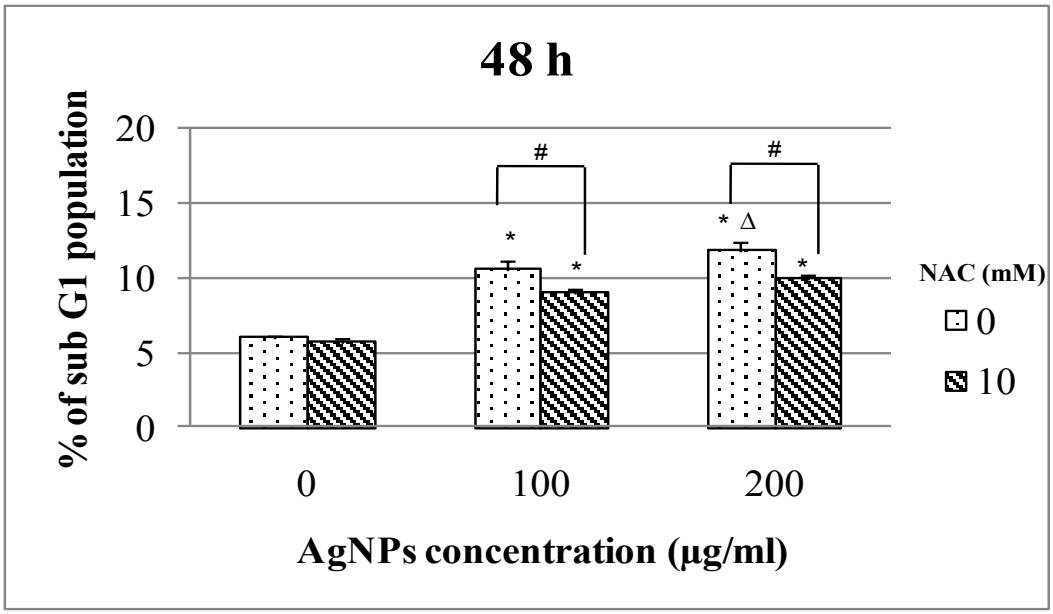


Figure 26 Effect of NAC on the change of subG1 population caused by AgNPs in A549 cells. Cells were treated with 10 mM NAC for 1 h and exposed to different concentrations (100, 200 μg/ml) of AgNPs for 48 h. Data shown are percentage of cells in subG1. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

Δ $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs without pretreatment of NAC

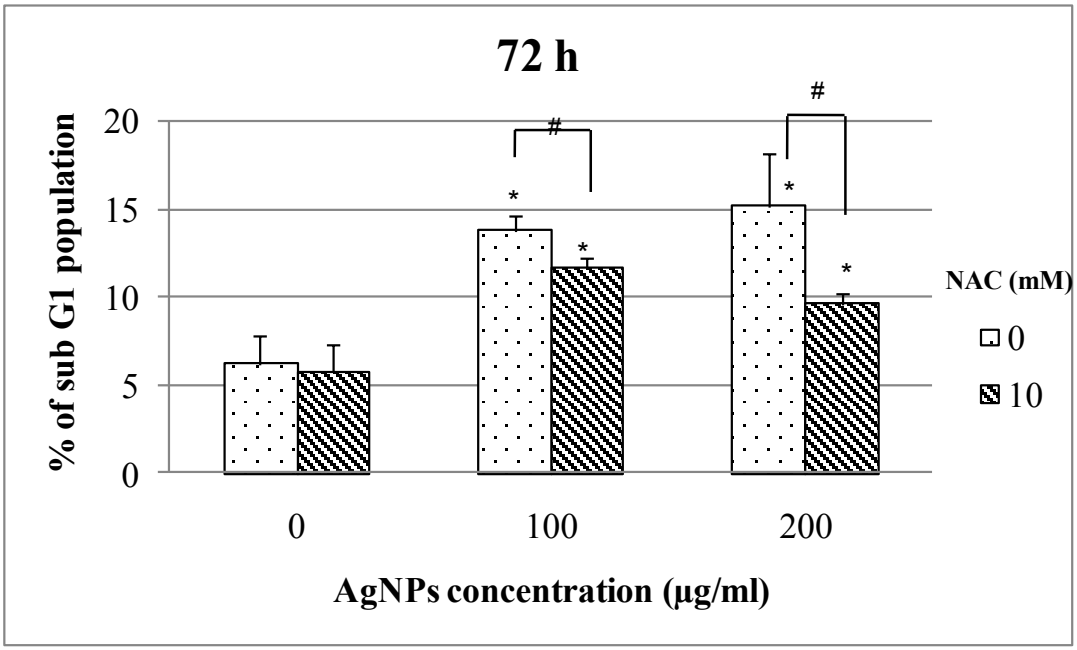


Figure 27 Effect of NAC on the change of subG1 population caused by AgNPs in A549 cells. Cells were treated with 10 mM NAC for 1 h and exposed to different concentrations (100, 200 µg/ml) of AgNPs for 72 h. Data shown are percentage of cells in subG1. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

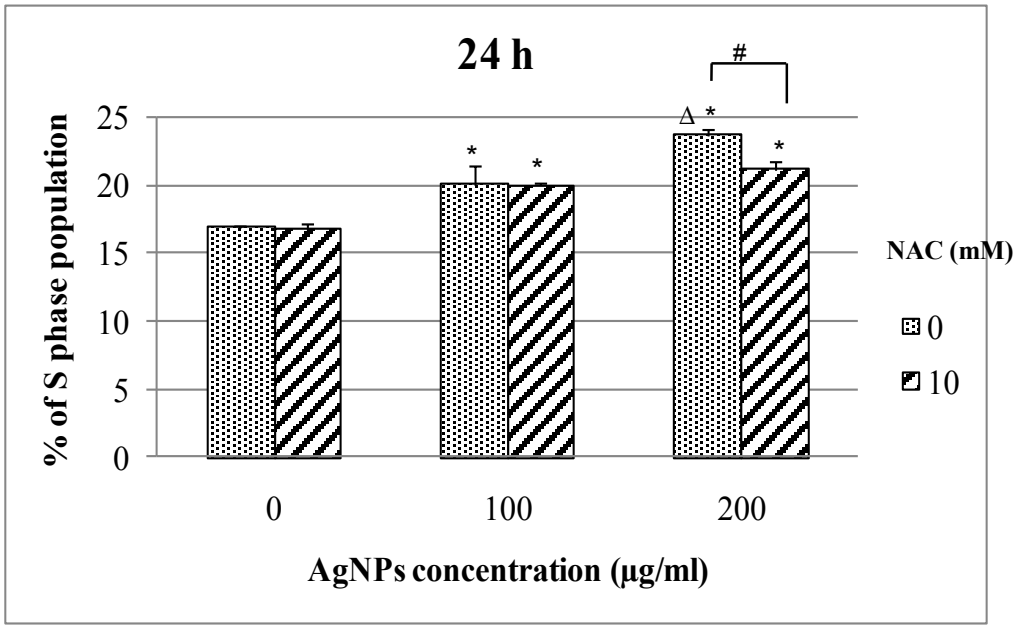


Figure 28 Effect of NAC on the change of S phase population caused by AgNPs in A549 cells. Cells were treated with 10 mM NAC for 1 h and exposed to different concentrations (100, 200 μg/ml) of AgNPs for 24 h. Data shown are percentage of cells in subG1. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

Δ $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs without pretreatment of NAC

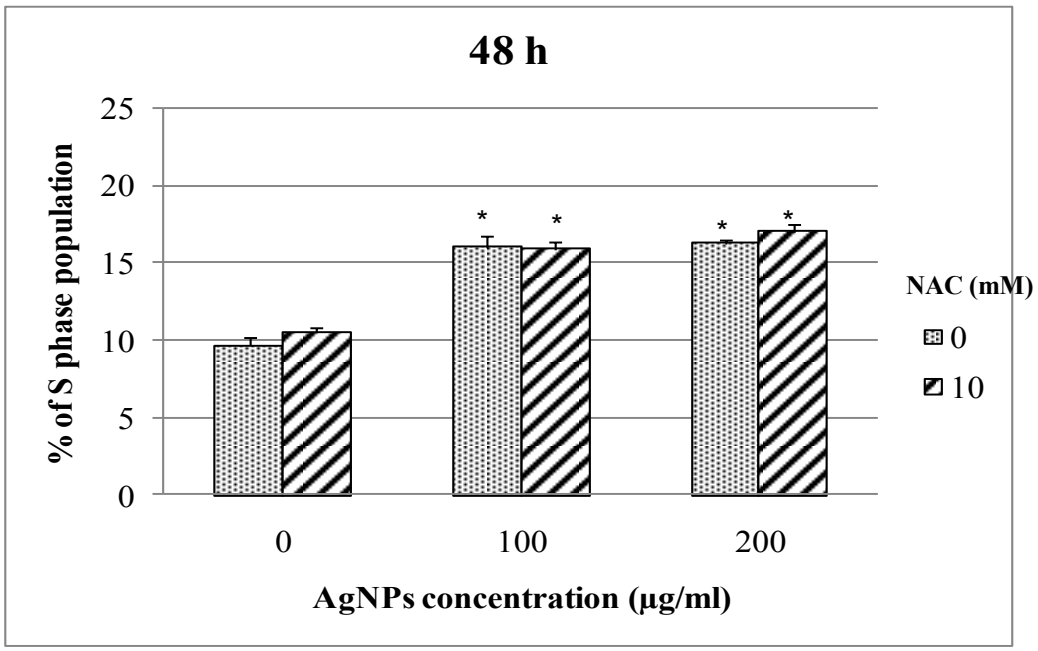


Figure 29 Effect of NAC on the change of S phase population caused by AgNPs in A549 cells. Cells were treated with 10 mM NAC for 1 h and exposed to different concentrations (100, 200 μg/ml) of AgNPs for 48 h. Data shown are percentage of cells in subG1. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

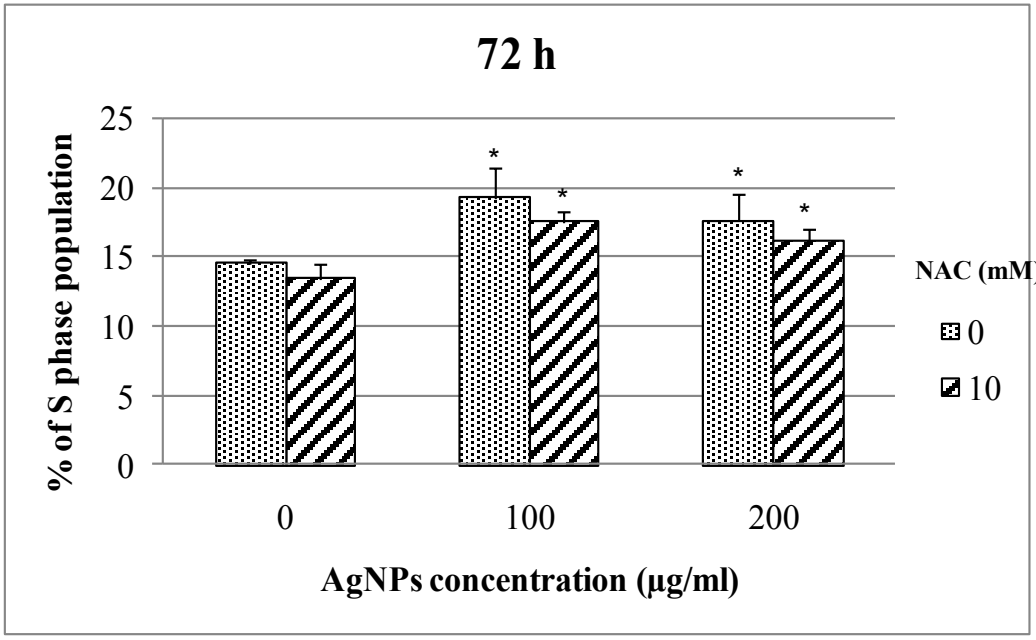


Figure 30 Effect of NAC on the change of S phase population caused by AgNPs in A549 cells. Cells were treated with 10 mM NAC for 1 h and exposed to different concentrations (100, 200 µg/ml) of AgNPs for 72 h. Data shown are percentage of cells in subG1. The data shown are mean ± SD of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; NAC (10 mM) pretreated group vs non-NAC (0 mM) pretreated group at the same concentration of AgNPs

7. Proliferating Cell Nuclear Antigen (PCNA) expression analysis

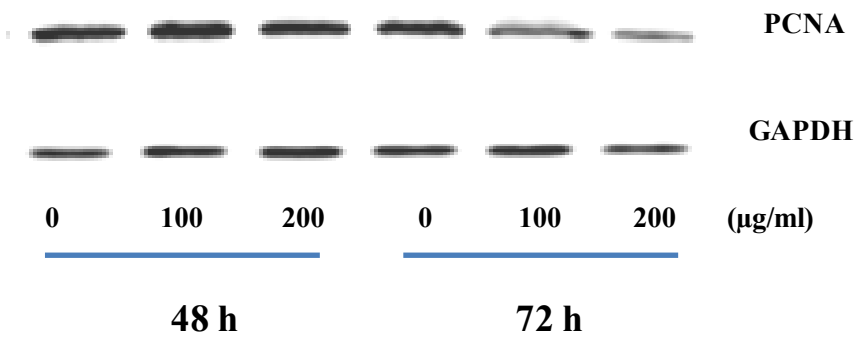
7.1 Effect of AgNPs on PCNA protein expression in A549 cells

To investigate effect of AgNPs on PCNA protein expression in A549 cells, cells were incubated in AgNPs at high concentrations (100 and 200 μg/ml) for 48 and 72 h and the change of PCNA protein expression after exposed to AgNPs was detected by Western blot analysis. As shown in Figure 31A, PCNA protein expression was significantly down-regulated by AgNPs exposure for 72 h in a concentration-dependent manner (100 and 200 μg/ml groups) but the effect was not shown at 48 h of AgNPs incubation. The band intensity of PCNA protein in the group treated with 100 and 200 μg/ml of AgNPs were decreased from 1 to 0.66 and 0.53 as compared to that of the control, respectively (Figure 31B).

7.2 Effect of antioxidant on the change of PCNA protein expression modulated by AgNPs in A549 cells

To evaluate whether NAC could prevent the change of PCNA protein expression modulated by AgNPs, cells were incubated with 10 mM NAC 1 h prior to AgNPs exposure. Then, cells were incubated with AgNPs at different concentrations (100 and 200 μg/ml) for 72 h and the change of PCNA protein expression was detected by Western blot analysis. The result was shown in Figure 32A. Analyzing from band intensity, AgNPs caused significant decrease of PCNA expression at both 100 and 200 μg/ml of AgNPs. However, NAC pretreatment did not attenuate the effect of AgNPs on the change of PCNA protein expression (Figure 32B).

(A)



(B)

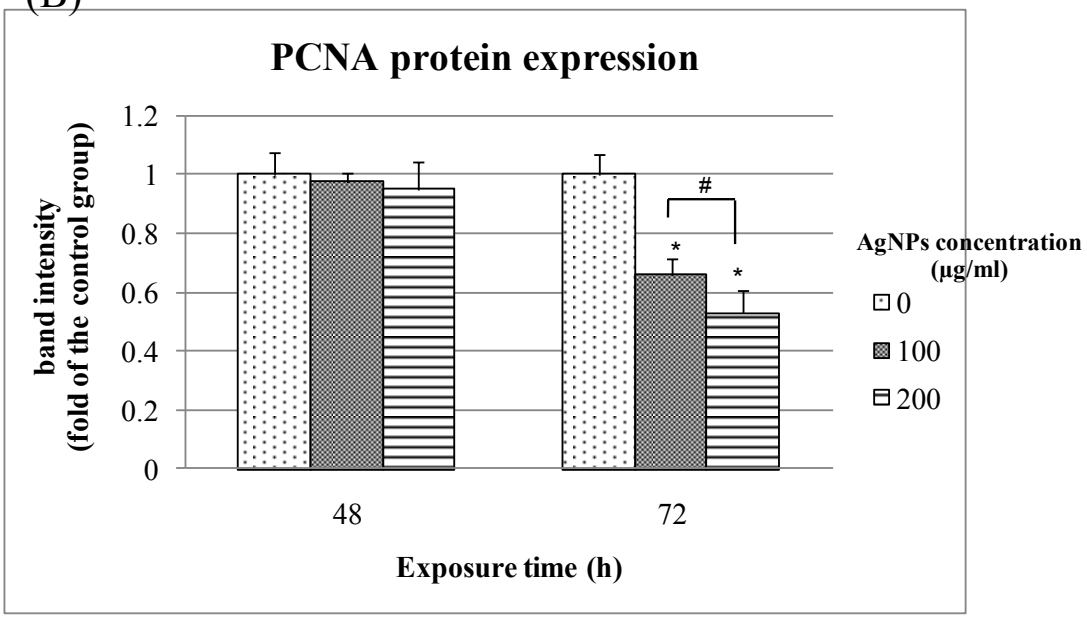


Figure 31 Effects of AgNPs on PCNA expression in A549 cells. (A) Representative blot of Western blot analysis of PCNA protein expression of A549 cells treated with AgNPs (100 and 200 μg/ml) for 48 and 72 h. (B) Mean ± SD of band intensity of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

# $P < 0.05$; 200 μg/ml of AgNPs vs 100 μg/ml of AgNPs

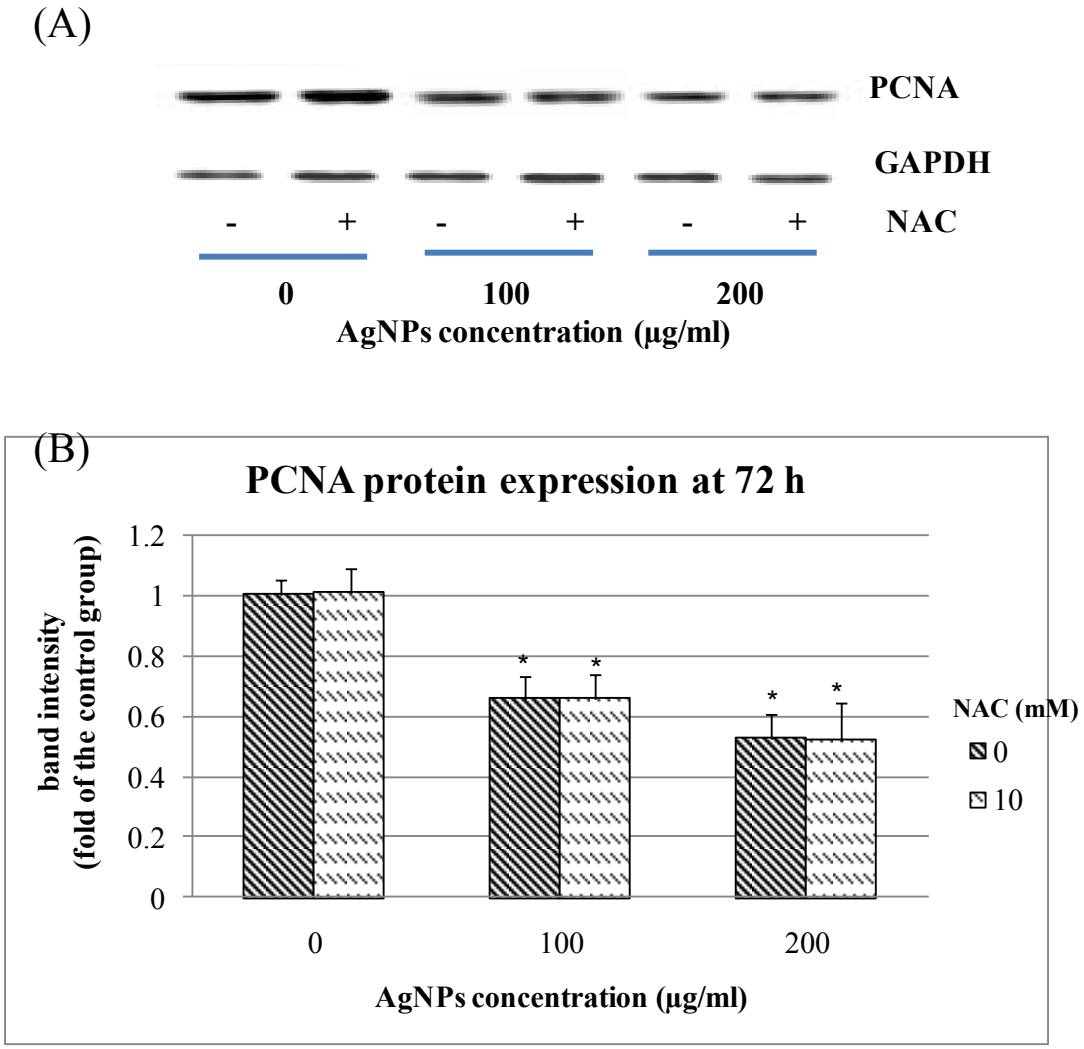


Figure 32 Effects of NAC on the change of PCNA expression modulated by AgNPs in A549 cells. (A) Representative blot of Western blot analysis of PCNA protein expression of the A549 cells treated with AgNPs (100 and 200 μg/ml) for 72 h. Cells were treated with 10 mM NAC for 1 h before exposure to different concentrations (100, 200 μg/ml) of AgNPs for 72 h. (B) Mean ± SD of band intensity of three independent experiments.

* $P < 0.05$; AgNPs treated group vs the corresponding control without AgNPs treatment

CHAPTER V
DISCUSSION AND CONCLUSION

Previous studies demonstrated that AgNPs generated ROS, abolished antioxidant enzymes such as glutathione peroxidase and superoxide dismutase leading to formation of DNA adducts (Arora *et al.,* 2008; Foldbjerg *et al.,* 2010). Moreover, *in vivo* studies revealed that AgNPs which were recognized as pulmonary toxicants potentially caused pathological changes in lung (Sung *et al.,* 2009; Cha *et al.,* 2008). So, we chose the A549 cells as an *in vitro* model system of human lung epithelial cells to investigate mechanism of AgNPs-induced pulmonary toxicity.

Silver nanoparticles used in this study were in non-coated powder form, so they have a tendency to aggregate in aqueous solution. In additions, AgNPs seemed to easily agglomerate or aggregate in the test media. For this reason, we improved their dispersion by sonication of 2 mg/ml AgNPs stock suspension in DI for 10 min and serial dilution in the media containing FBS. These particles demonstrated hydrodynamic diameter of about 180–190 nm when analyzed by DLS (Table 4). However, in particle characterization by TEM, the AgNPs showed spherical shape with primary particle diameter about 40–50 nm (Figure 14). Because of different techniques and assay conditions, the particle sizes or hydrodynamic diameter obtained from DLS method were larger than those from TEM method (Murdock *et al.,* 2008). Morphological examination of A549 cells displayed distinct cellular extensions in AgNPs-treated cells as compared to the control (Figure 15) which was in accordance to a previous study showing potentially disturbance of cytoskeletal functions caused by AgNPs (AshaRani *et al.,* 2009). However, apparently massive cell death was not observed. Accordingly, we performed cytotoxicity assessment to investigate the effects of AgNPs in the cells. The results from MTT assay, which was employed to detect mitochondrial reductase activity in living cells, illustrated concentration and time-dependent decrease of cell viability in the cells exposed to AgNPs (Figures 16 and 17) in accordance to previous study using PVP-coated AgNPs (Foldbjerg *et al.,* 2010). It indicated that AgNPs potentially affected cell survival by disturbing mitochondria.

As mentioned above, intracellular ROS is an important indicator to signify various toxic effects from nanoparticles (Arora *et al.,* 2008; Foldbjerg *et al.,* 2010). ROS bring about pro-oxidant environment in the cells, perturb a redox potential equilibrium and lead to adverse biological consequences ranging from early stage of inflammation to serious cell death (Maria *et al.,* 2007). Starch-coated AgNPs-mediated ROS generation is designated as a crucial factor for cytotoxicity (AshaRani *et al.,* 2009). In this study, we also investigated potential role of ROS generation by non-coated AgNPs in A549 cells using DCF assay. As expected, the results revealed a concentration-dependent increase of intracellular ROS in treated cells (Figure 18). It is known that NAC is a precursor of glutathione deactivating ROS system. Therefore, the effects of NAC to prevent AgNPs-induced ROS generation were also investigated. NAC was pretreated to cells before exposure to AgNPs and then subjected to MTT and DCF assays. The results showed that NAC not only reduced AgNPs-induced intracellular ROS generation but also improved cell viability after exposure to AgNPs (Figures 16-18) indicating that the particles were toxic to the cells via ROS generation. Our results confirmed the significance of ROS in AgNPs-induced toxicity as described earlier in AgNPs from the previous study of Foldbjerg *et al.,* (2010). Subsequently, we performed the further experiments to investigate the role of ROS in various aspects of AgNPs toxicity regarding changes of mitochondrial membrane potential and cell cycle progression as well as the expression of a cell cycle-associated protein, PCNA.

Mitochondria are known as cellular redox equilibrium-regulated organelles to maintain viability of the cells. They are a main source of cellular ROS metabolism (Kowaltowski *et al.,* 2009). Excess of ROS production can lead to the declination of MMP and then gives rise to uncontrolled ROS formation, vice versa. Results from TMRE assay (Figure 19) demonstrated the significant decrease of MMP in AgNPs treated cells. In this case, NAC could attenuate the declination of MMP only at 100 µg/ml AgNPs, but not 200 µg/ml AgNPs, for up to 72 h (Figures 20-22). Therefore, we presumed that ROS was partially involved in MMP collapse in AgNPs treated cells.

Previous reports indicated that nanoparticles can induce apoptosis through oxidative stress-mediated pathway (AshaRhani *et al.,* 2010 and Foldbjerg *et al.,*

2010). Our FACS analysis of cell cycle revealed the increment of subG1 population in AgNPs-treated cells in a concentration and time-dependent manner (Figure 23). This study pointed out that apoptosis might occur after exposure to AgNPs. In addition, we found that the increase of Sub G1 was mostly prevented by NAC pre-treatment as shown in Figures 25-27. Therefore, the results suggested that the apoptotic effect was associated with generation of ROS.

The early effect of AgNPs will be apparently seen in cell cycle progression. It was recently informed that carbon black coated with benzo(α)pyrene induced S phase arrest in human lung epithelial cells (Mroz *et al.,* 2007). Additionally, G2/M phase arrest was investigated in human glioblastoma cells and human lung fibroblasts after exposure to starch-coated AgNPs (AshaRani *et al.,* 2009). In general, toxic substances which perturb the cell cycle progression commenced at retardation of cell growth (anti-proliferative effect) followed by accumulation of cells population in some phases of cell cycle, such as S or G2/M phase arrest. Higher level of toxicity could be identified as a prominent decrease of cell viability and eventually leading to cell death (cytotoxicity effect).

Also, cells treated with compounds which have a competency to inhibit DNA synthesis could exhibit S phase arrest (Black *et al.,* 1989). In this study, the influence of non-coated AgNPs on changes of cell cycle was analyzed by PI staining followed by FACS analysis. In treated cells, a corresponding decrease in G1 population accompanied by an increase in S phase population was detected (Figure 24). In contrast, non-coated AgNPs had a little impact on G2/M phase. Interestingly, no statistical significant difference of S-phase population was observed between with or without NAC pretreatment (Figures 28-30). These results indicated that reduction of ROS by NAC could not prevent S phase arrest. In conclusion, anti-proliferative effect of AgNPs resulting from induction of S phase arrest and this effect was possibly associated with only AgNPs themselves, not directly caused by ROS.

To further investigate mechanism of non-coated AgNPs-induced S phase arrest, expression of a cell cycle-associated protein, PCNA, was measured. Among cell cycle-associated proteins, PCNA is an interesting protein containing versatile functions in various steps of cell cycle (Maga and Hübscher, 2003). PCNA is a protein synthesized in the early G1 and S phases of the cell cycle and responsible for

cell cycle regulation, DNA replication and DNA repair (Strzalka and Ziemienowicz, 2010). Formerly, PCNA has been found in the nuclei of cells that undergo cell division (Maga and Hübscher, 2003). From the recent study, PCNA exerts three functions such as a sliding clamp operator during DNA synthesis, a polymerase switch factor and a recruitment factor of DNA polymerase δ (Maga and Hübscher, 2003). PCNA down-regulation may indicate cell cycle arrest (Engel *et al.,* 2003). NIH guideline for investigating proliferative effects of nanoparticles, stated that immunostaining of PCNA correlates well with other cell proliferation detection methods (Jedd *et al.,* 2010) Our results demonstrated the relation between PCNA expression (Figure 31) and S-phase arrest (Figure 24). Surprisingly, Western blot analysis demonstrated that PCNA expression was significantly down-regulated by AgNPs exposure in a concentration and time-dependent manner, as shown in Figure 31. In addition, as shown in Figure 32, NAC could not prevent down-regulation of PCNA expression in AgNPs-treated groups. This observation was well-correlated with the S-phase arrest which showed no statistical significant difference between NAC-pretreated groups and that of the controls (Figures 28-30). Another cell cycle regulated protein, which has a strong relationship to PCNA is p21. This protein is well-known as DNA replication associated protein which blocks progression from S to G2 phase of the cell cycle (Strzalka and Ziemienowicz, 2010). PCNA is considered as an essential mediator of the regulatory action of p21 (Waga *et al.,* 1994). Binding of p21 to PCNA results in inhibition of DNA replication (Podust *et al.,* 1995; Shivji *et al.,* 1992). Furthermore, the high levels of p21 and down regulation of PCNA can lead to cell cycle arrest (Engel *et al.,* 2003). In this regard, a possible mechanism for anti-proliferative effect of non-coated AgNPs, may be explained by inhibition of PCNA expression, which might resulting in the high levels of p21, leading to inhibition of cell cycle progression from S to G2 phase, which referred to as cell cycle arrest. The relationship between p21 and PCNA expressions in AgNPs-induced cell cycle arrest should be further investigated.

Taken together, these observations provide a possible scenario of cytotoxicity of non-coated AgNPs in A549 cells. Non-coated AgNPs possibly cause toxicity to A549 cells via at least, two major pathways. One is ROS-dependent pathway (cytotoxicity) which is directly relevant to ROS. Starting from AgNPs potentially

generate ROS in cells where ROS bring about various toxic effects. Among these effects, disturbance of normal MMP, mitochondria function and apoptosis were significantly related to the intracellular ROS level. Another one is anti-proliferative effect which is an ROS-independent pathway. Non-coated AgNPs exhibit this toxic effect by themselves without an association with ROS.

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APPENDICES

APPENDIX A
POSTER PRESENTATION

This research work was presented as a poster presentation, entitled TOXICITY OF SILVER NANOPARTICLES IN A549 CELLS, at 33rd Pharmacological and Therapeutic Society of Thailand Meeting, Prince of Songkla University, Songkla on 17-19 March, 2011. The poster number of participant was 26.

TOXICITY OF SILVER NANOPARTICLES IN A549 CELLS

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I. INTRODUCTION

The rapid advancement of nanotechnology leads to producing of a vast array of engineered nanoparticles (NPs) which are defined as materials measured in length or diameter within the range of 1-100 nm in at least one dimension. With reduction in size, specific surface area and reactivity of NPs dramatically increase. It has been reported that these novel physicochemical properties bring NPs to be bioreactive species when accessing into living organisms (1).

Silver nanoparticles (AgNPs) have gained considerable attention as compared to other metal nanomaterials because of their attractive physicochemical properties. AgNPs are nowadays incorporated in a large number of consumer and medical products (2). Several experiments demonstrated that nano-sized silver can be toxic to human's vital organs especially lung which is found to be the least protective exposure route. AgNPs not only bind proteins contained –SH groups with strong affinity but also persistently accumulate in the lung for many years (3).

Although one of the toxicities of AgNPs is to mediate oxidative stress (4), mechanism of AgNPs toxicity at the cellular and molecular level has not yet been fully explained. Thus, the aim of this study was to investigate cell viability and change of cell cycle in response to AgNPs treatment in order to elucidate a possible mechanism of AgNPs-induced pulmonary toxicity.

II. MATERIALS AND METHODS

Materials

AgNPs, in an amorphous form, were purchased from Sigma (St. Louis, MO). The product information indicates that particle sizes were less than 100 nm and specific surface areas were 5.0 m^2/g.

Cell lines and cell culture

Human lung carcinoma (A549) cells were obtained from ATCC (Manassas, VA) and grown in F-12K media supplemented with 10% fetal bovine serum, in a 95% humidified atmosphere and 5% CO_2 at 37°C.

Particle Characterization

The particles were characterized by transmission electron microscope (TEM; JEM-2010, Jeol).

In vitro cytotoxicity assay

Cytotoxicity was assessed by using MTT, a mitochondrial-based cell viability assay. A549 cells ($5x10^3$ cells/well) were seeded in 96-well plates. After 24 h, the cells were treated with 0, 25, 50, 100, 200 µg/ml of AgNPs for 24 and 48 h. Subsequently, MTT reagent was added to the cells and further incubated for 1 h. The resulted formazan crystal was dissolved with DMSO. Absorbance of the solution was measured at a wavelength of 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

Cell cycle analysis

The cells were cultured in media containing 0, 50 and 100 µg/ml of AgNPs for 24 and 48 h. At the end of treatment, the cells were harvested, fixed with 80% ethanol for 30 min at 4°C, and stained with 50 µg/ml propidium iodide in the presence of 0.2 mg/ml RNaseA for 30 min at room temperature. The cell suspension was filtered by a nylon mesh filter, and the filtrate was subjected to FACS analysis using a MoFlo cell sorter analyzer (Dako Cytomation, Kyoto, Japan).

DCF assay

Intracellular ROS after AgNPs treatment was measured by using 2',7'-dichloroflourescein diacetate (DCFH-DA) which reacts with ROS. The final reaction generates a flourescent compound dichloroflourescein (DCF). The flourescent intensity was detected by using a microplate flourometer at excitation and emission wavelength of 485 and 528 nm, respectively. SIN-1 were used as positive controls.

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III. RESULTS

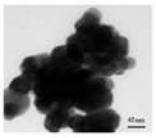

Figure 1. A TEM image of silver nanoparticles.

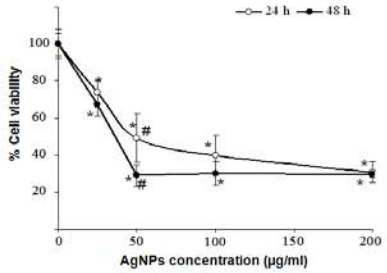

Figure 2. Percent cell viability of A549 cells after treatment with different concentrations of silver nanoparticles for 24 and 48 h * $p < 0.05$; AgNPs treatment vs control and # $p < 0.05$; 24 h vs 48 h

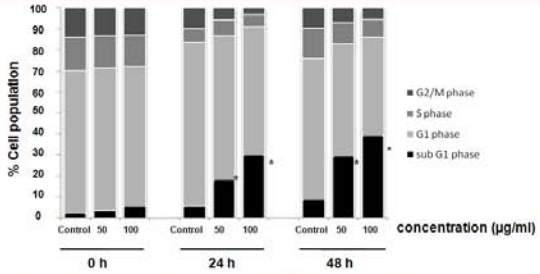

Figure 3. A concentration-dependent sub G1 arrest after A549 cells exposed to AgNPs for 24 and 48 h * $p < 0.05$; AgNPs treatment vs control

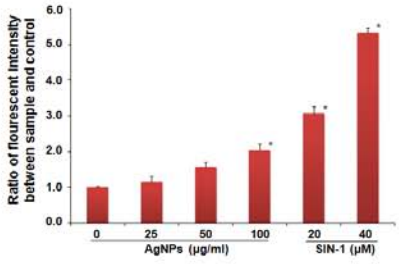

Figure 4. A concentration-dependent intracellular ROS after A549 cells exposed to AgNPs and SIN-1 for 3 h * $p < 0.05$; AgNPs treatment vs control

IV. CONCLUSIONS

Results of this study demonstrated the toxicological effect of AgNPs on A549 cells which included interfering of mitochondrial function, disturbing of normal cell cycle. Therefore, this study demonstrated that AgNPs have a potential to initiate pulmonary toxicity.

สมาคมเภสัชวิทยาแห่งประเทศไทย

ร่วมกับ

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นางสาวพรทิพา ไชยเรืองกิตติ

ได้เข้าร่วมการประชุมและเสนอผลงานในการประชุมวิชาการประจำปี
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