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In Vitro Toxicity of Nanoparticles in Mouse Keratinocytes and Endothelial Cells

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THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

FOR THE DIRECTOR

//signed//

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14. ABSTRACT
This study was undertaken to assess the potential toxicity of nanomaterials in mouse keratinocytes (HEL-30) and endothelial cells (bEnd.3). The nanoparticles tested were aluminum (Al – 30 nm), silver (Ag – 15 nm hydrocarbon coated, 100 nm uncoated), and molybdenum trioxide (MoO3 – 30 nm). For toxicity evaluations, mitochondrial function (MTT assay), lysosomal membrane integrity (NR assay) and cellular morphology were assessed under control and exposed (10 – 250 mg/ml) conditions for an exposure period of 24 hours. In the MTT assay, the 15 nm Ag particles were found to be highly toxic in HEL-30 cells when compared to Al-30 nm and MoO3-30 nm particles, which produced no toxicity at the tested concentrations. The 100 nm Ag particles also did not produce significant toxicity in HEL-30 cells. However, Ag-100 nm particles did induce toxicity in bEnd.3 cells. bEnd.3 cells were also three times more sensitive to Ag-15 as compared to HEL-30 cells. Like HEL-30 cells, the bEnd.3 cell line displayed no toxicity in the presence of Al-30 nm or MoO3-30 nm particles. NR uptake data in HEL-30 cells also confirmed that Ag-15 nm particles were highly toxic compared to Al and MoO3 nanoparticles. Observation using a phase contrast inverted microscope indicated that increased concentration of Ag-15 led to a change in cell morphology. Cells affected by nanoparticle exposure showed a decrease in cellular volume and a change in cell shape. Further comparisons of other nanomaterials are planned using in vitro cells originating from pulmonary, liver and skin tissues.

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Nanoparticles, aluminum, molybdenum trioxide, silver, in vitro, mitochondrial function, lysosomal membrane integrity, cell morphology, keratinocyte cell line (HEL-30)

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PREFACE

This technical report describes experimental laboratory work conducted by the U.S. Air Force, air Force Research Laboratory. Applied Biotechnology Branch (AFRL/HEPB). The research began January 2003 and was completed in June 2004. A portion of the project was completed under Department of the Air Force Contract No. F33615-00-C-6060. Dr. David Mattie served as the Contract Technical Monitor for AFRL/HEPB and Dr. Darol Dodd served as Program Manager for the ManTech/GEO-CENTERS Joint Venture Contract (F33615-00-C-6060). ManTech Environmental Technology, Inc. was acquired by Alion Science and Technology Corporation during this project.

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IN VITRO TOXICITY OF NANOPARTICLES IN MOUSE KERATINOCYTES AND ENDOTHELIAL CELLS

EXECUTIVE SUMMARY

Skin epidermal cells include keratinocytes, endothelial cells and fibroblasts. Keratinocytes form the stratified layers of the epidermis and serve as a protection barrier for environmental threats, such as nanoparticles. A nanoparticle that induces a toxic effect in keratinocytes is thus a danger to endothelial cells and fibroblasts as well. In light of the significant toxic effect that silver has on keratinocytes, the present study was undertaken to assess the potential toxicity of nanomaterials in a mouse keratinocyte cell line (HEL-30) and endothelial cell line (bEnd.3). Aluminum (Al – 30 nm), silver (Ag – 15 nm hydrocarbon coated, 100 nm uncoated), and molybdenum trioxide (MoO_3 –30 nm) nanoparticles were tested in this toxicity study. Initially, a dispersion assessment of nanomaterials was conducted in aqueous (deionized water) and physiological (PBS) solutions in order to obtain homogenous suspension for exposures to cells. The dispersion test showed that Al and MoO_3 nanoparticles dispersed homogenously in PBS. In contrast, Ag nanoparticles showed a slight precipitation in PBS; thus, Ag was dispersed in de-ionized water. Endpoints for toxicity evaluations included mitochondrial function (MTT assay), lysosomal membrane integrity (NR assay) and cellular morphology; these were assessed under control and exposed conditions (10 - 250 μg/ml) for an exposure period of 24 hours. In order to compare the toxicity of nanoparticles, cadmium oxide (CdO – 1000 nm) was used as a positive control; CdO was highly toxic with an effective concentration producing 50% cell death (EC_{50}) of 1.63 ± 0.03 μg/ml in both HEL-30 and bEnd.3 cells. Titanium dioxide (TiO_2 - 40 nm) was used as a negative control; no toxicity was observed at all tested concentrations in either cell line (10 - 250 μg/ml).

In the MTT assay, the 15 nm Ag particles were found to be highly toxic in HEL-30 cells when compared to Al-30 nm and MoO_3-30 nm particles (EC_{50} of >250 μg/ml for both). The EC_{50} value for Ag-15 nm particles was 35.4 μg/ml. Based on this EC_{50} value, Ag-15 nm particles were seven times more toxic than Al and MoO_3 particles in HEL-30 cells. The 100 nm Ag particles did not produce significant toxicity in HEL-30 cells. However, Ag-100 nm particles did induce toxicity in bEnd.3 cells (EC_{50} 100.0 ± 2.5 μg/ml). bEnd.3 cells were also three times

more sensitive to Ag-15 as compared to HEL-30 cells (EC50 of 10.8 ± 2.5 vs. 35.4 ± 7.6 μg/ml, respectively). Like HEL-30 cells, the bEnd.3 cell line displayed no toxicity in the presence of Al-30 nm or MoO_3-30 nm particles (EC_{50} of >250 μg/ml for both). NR uptake data in HEL-30 cells also confirmed that Ag-15 nm particles were highly toxic compared to Al and MoO_3 nanoparticles. Observation using a phase contrast inverted microscope indicated that increased concentration of Ag-15 led to a change in cell morphology. Cells affected by nanoparticle exposure showed a decrease in cellular volume and a change in cell shape. Further comparisons of other nanomaterials are planned using *in vitro* cells originating from pulmonary, liver and skin tissues.

1.0 – INTRODUCTION

Nanotechnology has contributed significantly in the development of diverse areas including renewable energy, revolutions in medicine, advanced techniques in security and crime detection, new approaches to tissue engineering and medical implants. The latest application in sunscreens and cosmetics, space travel and exploration attracted great attention by scientists for further investigations to alter these nanosize materials. Nanomaterials, which range in size from 1 - 100 nanometers, have been used to create unique devices at the nanoscale level possessing novel physical and chemical functional properties (Colvin, 2003; Oberdorster, 2004). Recent advancements in nanotechnology have facilitated the development of many new products such as decontamination systems (polymer gels containing nanoparticles), electromechanical systems (lab-on-a-chip devices) and florescent tags (contrast agents for magnetic resonance imaging).

These recent advancements in nanotechnology have inspired a renewed look at the requirements of the military and the role that nanomaterials can play to enhance the protection of personnel from environmental and adversary threats. The military has capitalized on the surge in nanotechnology development, namely in the areas of electronics, sensors and munitions. Despite the many benefits of nanotechnology, studies indicate that certain nanoparticles may cause adverse effects due to their small size, increased surface area relative to volume and increased surface energy. Human exposure during handling and release of nanoparticle materials into the environment upon detonation has not been studied significantly. Adverse health effects resulting from nanoparticle exposure needs to be investigated in order to ensure the safety and continued health of personnel involved in nanotechnology development and use of equipment and munitions designed with nanomaterials. The major toxicological concerns are that some of the manufactured nanomaterials are redox active (Colvin, 2003) and some particles transport across cell membranes, especially into mitochondria (Foley *et al.*, 2002). One of the few relevant studies was with single-wall carbon nanotubes in mice. Lam *et al.* (2004) demonstrated that carbon nanotube products induced dose-dependent epithelial granulomas in mice and, in some cases, interstitial inflammation in the animals of the 7-day post-exposure groups. The recent study by Oberdorster (2004) indicated that nanomaterials (Fullerenes C_{60}) induced oxidative stress in a fish model.

The use of *in vitro* methods for cytotoxicity studies has resulted in decreased costs due to the elimination of animals for exposures. *In vitro* models are rapid and convenient for initial studies to screen for nanomaterial toxicity. The results from these studies can facilitate future decisions on whether or not to advance to an *in vivo* model as a basis for further assessing the potential risk of nanomaterial exposure and health effects. The HEL-30 immortal mouse keratinocyte cell line was selected as the working model system to screen, at low cost and in a relatively short time frame, for the general toxicity of several nanomaterials.

Skin epidermal cells include keratinocytes, endothelial cells and fibroblasts. Keratinocytes form the stratified layers of the epidermis and serve as a protection barrier for environmental threats, such as nanoparticles. A nanoparticle that induces a toxic effect on keratinocytes is thus a danger to endothelial cells and fibroblasts as well. Due to the fact that skin contains endothelial cells, an immortalized mouse endothelial cell line (bEnd.3) was selected as the working model system for initial endothelial toxicity testing. The toxicological tests performed for viability were the neutral red (NR) assay, which measures cytoplasmic lysosomal membrane integrity, and the mitochondrial reduction of the tetrazolium salt 3-(4, 5-dymethylthiazol-2-yl)-2, 5-dyphenyltetrazolium bromide (MTT), which analyzes mitochondrial function (MTT assay).

2.0 – MATERIALS & METHODS

2.1. Nanomaterials:

The nanoparticles (Al – 30 nm; MoO3 – 30 nm; Ag – 15 nm with carbon coating, 100 nm without coating) were provided by Dr. Kiel, Air Force Research Laboratory, Brooks City Base, TX. Cadmium oxide (CdO-1000 nm) and titanium oxide (TiO_2-40 nm) were purchased from Fluka Chemicals and Altair, Nanomaterials Inc, respectively. Limited details are currently available on the chemical properties of these materials.

2.2. Chemicals:

The 3-(4,5-dimethylthiazol-2-yl)-2,5-dyphenyltetrazolium bromide (MTT), penicillin-streptomycin, Neutral Red (NR) solution and Ham's Nutrient Mixture F-12 media were purchased from Sigma Chemical Company (St. Louis, MO). The fetal bovine serum (FBS) used in the media was from Gibco^{TM} Invitrogen Corporation (Carlsbad, CA).

2.3. Dispersion Tests – Dispersion of Nanomaterials in Solution:

The dispersion tests were conducted in physiological phosphate buffered saline (PBS) or deionized water. Using physical mixing and sonication, stock solutions were prepared either in PBS or deionized water. The stock solutions of MoO_3 (30 nm), TiO_2 (40 nm), CdO (1000 nm) and Al (30 nm) were prepared in PBS (Figure 1) while Ag (15 nm, 100 nm) were prepared in deionized water (Figure 1). Preceding exposure, diverse concentrations of nanoparticles were dissolved in Ham's Nutrient Mixture F-12 media that did not contain FBS.

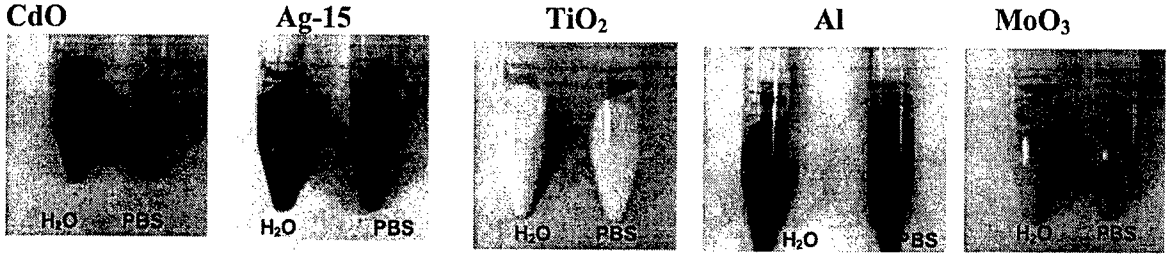

Figure 1: Dispersion of nanoparticles in aqueous and physiological solutions

2.4. Cell Culture:

HEL-30 immortalized mouse keratinocytes were used between passages 28 and 32. HEL-30 cells were grown in Ham's Nutrient Mixture F-12 with 5% FBS. Cells were plated on collagen coated 6-well plates for 48-72 hours until they became confluent preceding any dosing with nanoparticles. The cells were maintained in a 5% CO_2 incubator at 35°C.

The bEnd.3 immortalized mouse endothelial cells were used between passages 5 and 7. The bEnd.3 cells were also grown in Ham's Nutrient Mixture F-12 with 5% FBS and plated on collagen coated 6-well plates for 48-72 hours until confluent prior to any dosing. Cells were maintained in a 5% CO_2 incubator at 35°C.

2.5. Treatment and Toxicity Endpoints:

Once the monolayer of cells was established, within 95-100% confluence, HEL-30 and bEnd.3 cells were exposed to a range of nanoparticles and concentrations. The nanoparticle concentrations were suspended in Ham's Nutrient Mixture F-12 media without FBS. The dosing period for each exposure was 24 hours. After the 24 hour treatment, toxicity endpoints (MTT, NR and external morphology) were evaluated in control and nanoparticle-exposed cells (Figure 2).

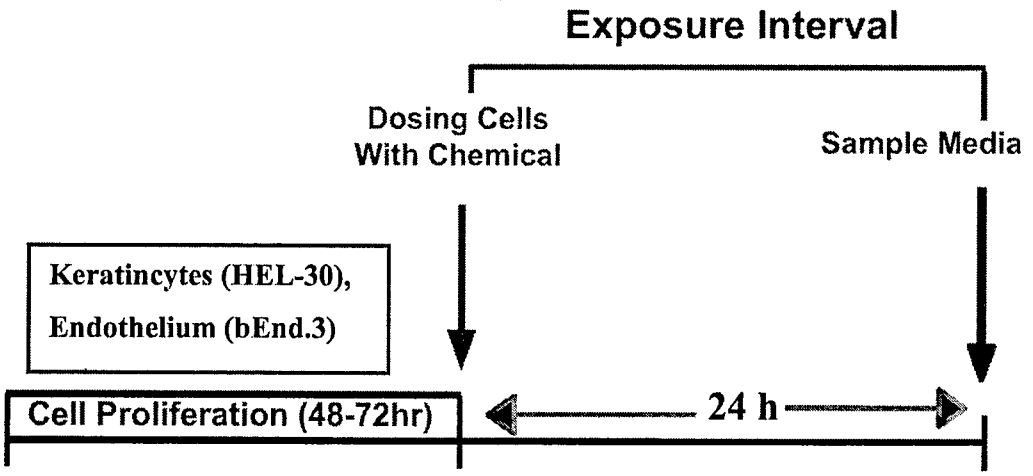

Figure 2: Timeline for growth of keratinocytes (HEL-30) and endothelial cells (bEnd.3) and subsequent exposure to nanoparticles

2.5.1 – MTT Assay-Mitochondrial Function:

Mitochondrial function was evaluated in HEL-30 and bEnd.3 cells spectrophotometrically by measuring the degree of mitochondrial reduction of the tetrazolium salt, 3-(4, 5-dymethylthiazol-2-yl)-2, 5-dyphenyltetrazolium bromide (MTT), to formazan by succinic dehydrogenase (Carmichael *et al.*, 1987). Following treatment, cells were washed and incubated at 35°C in Chee media containing a 1:10 dilution of MTT for 30 minutes. After 30 minutes incubation, the media containing MTT was removed and 1 ml of acidified isopropanol solution was added to each well. The plates were then placed on a plate shaker for 15 minutes. Two hundred µl of the homogenate were transferred from each well to a 96-well microplate and the absorbance (optical density, OD) was read at 550 nm with 650 nm as the reference on a SpectraMAX Plus 190 microplate reader (Molecular Devices, Sunnyvale, CA). Cytotoxicity was evaluated as follows:

1. Calculate the average of the absorbance (OD) values from triplicate samples.
2. Determine cytotoxicity using the following equation.

$$\%\ Cytoxicity = \frac{OD\ Values\ in\ Treated\ Cells}{OD\ Values\ in\ Control\ Cells} \times 100$$

2.5.2. The Neutral Red Cytotoxicity Assay:

Cytoplasmic vacuolar membrane leakage, specifically that of the lysosomes, was evaluated in HEL-30 cells spectrophotometrically by measuring the degree of lysosome uptake of the neutral red dye (3-amino-7-dymethlyamino-2-methylphenazine hydrochloride). Lysosomes of viable cells accumulate the dye. During cell death or membrane degradation, the red dye is no longer retained. Following treatment, cells were washed and incubated at 35°C in Ham's media containing a 1:100 dilution of NR for 30 minutes. After 30 minutes incubation, the media containing NR was removed and 1 ml of acidified isopropanol solution was added to each well. The plates were then placed on a plate shaker for 15 minutes. Two hundred µl of the homogenate were transferred from each well to a 96-well microplate and the absorbance was

read at 540 nm with a SpectraMAX Plus 190 microplate reader. This assay was not conducted for bEnd.3 cells due to time constraints. Cytotoxicity was evaluated as described in Section 2.5.1.

2.5.3. Qualitative Observation of External Morphology of Control and Exposed Cells by Phase Contrast Inverted Microscopy:

HEL-30 and bEnd.3 cells were exposed, as mentioned previously, at various concentrations of nanoparticles for 24 hours. At completion of the exposure period, cells were washed with PBS at room temperature and observed by phase contrast inverted microscopy at 10X magnification.

2.6. Statistical Evaluation:

The data were expressed as mean ± standard deviation (SD) of three independent experiments. Where appropriate, the data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Dunnett's Method for multiple comparisons (Hussain and Frazier, 2003). A value of $p < 0.05$ was considered significant. SigmaStat for Windows Version 2.03 software was used for the statistical analysis.

3.0 – RESULTS & DISCUSSION

3.1. Physical observation on dispersion of nanoparticles:

As shown in Figure 1, aluminum, molybdenum, cadmium and titanium dispersed homogenously in PBS. Silver precipitated slightly in PBS and therefore was dissolved in deionized water where it dispersed homogenously. As a result, PBS was selected for aluminum,

molybdenum, cadmium and titanium stock solutions for dosing; deionized water was used for silver. From the stock solutions, various final concentrations were prepared in cell growth medium prior to exposure. It was noted that cloudiness increased with increasing concentration of nanoparticles (Figure 3, 4). The cloudiness intensified significantly at 250 µg/ml in all of the nanoparticle solutions. Another physical observation with silver dispersion was that Ag-100 rapidly settled out of solution suspension and constant mixing was necessary before adding it to the Ham's media for exposure. Constant mixing was also necessary during exposure with the Ham's-Ag solution. This may be a combination of the lack of carbon coating and the larger size of the particles.

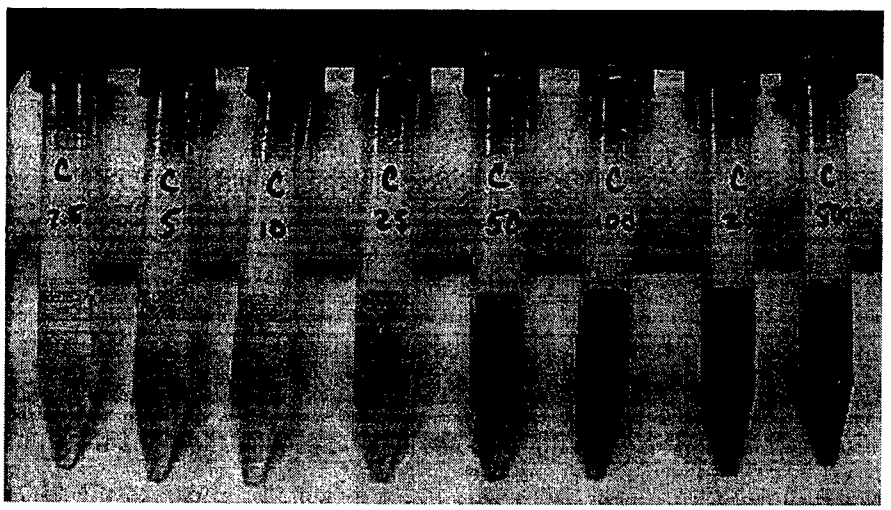

Figure 3: Different concentrations of silver -15 nm (2.5-500 µg/ml) in Ham's media before exposure

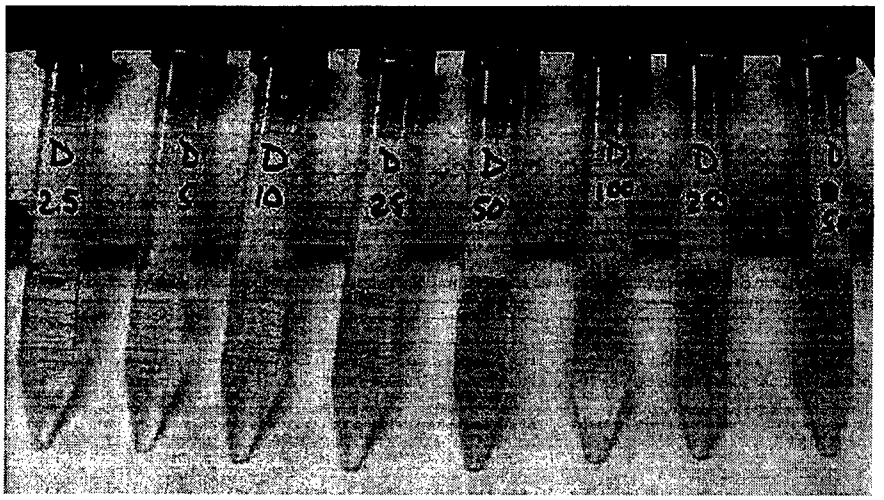

Figure 4: Different concentrations of silver -100 nm (2.5-500 µg/ml) in Ham's media before exposure

3.2. Cytotoxicity of nanoparticles:

A toxic dose range assessment study was conducted to determine the cytotoxicity resulting from nanoparticles exposure. In this study, HEL-30 cells were exposed to nanoparticles at concentrations ranging from 10 – 250 µg/ml for 24 hours. At the end of the 24 hour exposure period, cytotoxicity was evaluated using MTT reduction and NR reduction as endpoints. The qualitative external morphology of control and exposed cells was assessed by phase contrast inverted microscopy. Mitochondria are vulnerable targets for toxic injury by a variety of compounds because of their crucial role in maintaining cellular structure and function via aerobic ATP production (Hussain and Frazier, 2003).

A positive control (CdO) and negative control (TiO_2) were used for comparison with the test materials. Al-30 nm and MoO_3-30 nm did not produce significant toxicity in HEL-30 cells (Figure 5). Exposure to Ag-15 nm resulted in significant mitochondrial cytotoxicity from 25-250 µg/ml in HEL-30 cells (Figure 6) and from 25 – 250 µg/ml in bEnd.3 cells (Figure 7). The 100 nm Ag particles did not produce significant toxicity in HEL-30 cells (Figure 6). However, Ag-100 nm particles did induce cytotoxicity in bEnd.3 cells (Figure 7).

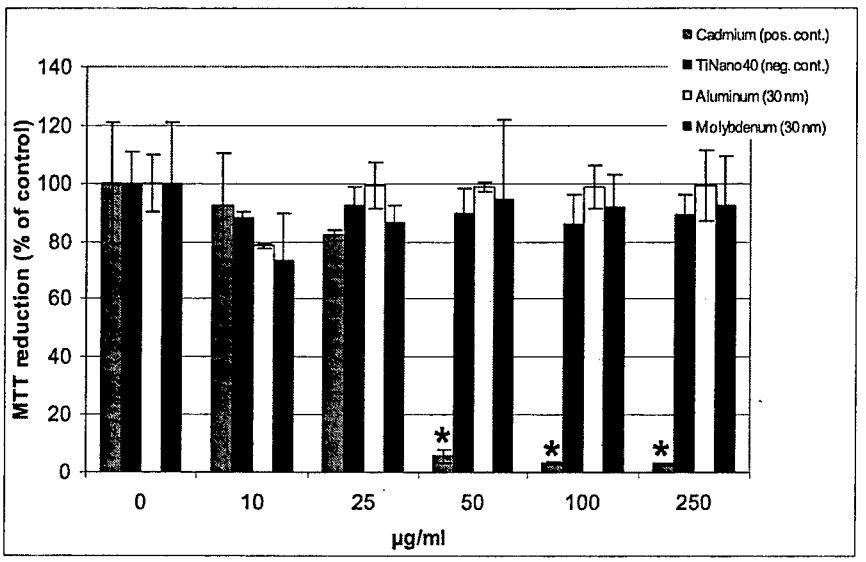

Figure 5: Effects of nanoparticles (24 hr exposure) on MTT reduction in HEL-30 mouse keratinocyte cell line. *Significantly different from control (0 µg/ml).

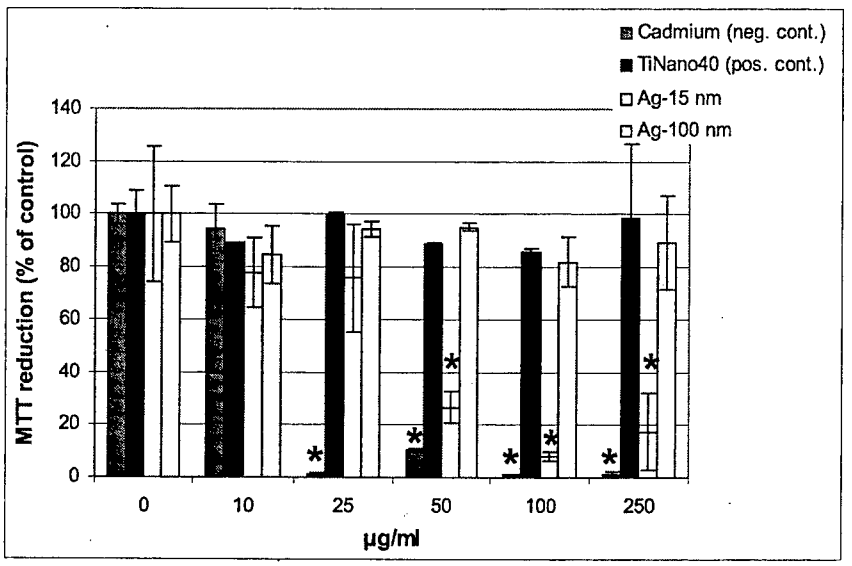

Figure 6: Effects of nanoparticles (24 hr exposure) on MTT reduction in HEL-30 mouse keratinocyte cell line. *Significantly different from control (0 µg/ml).

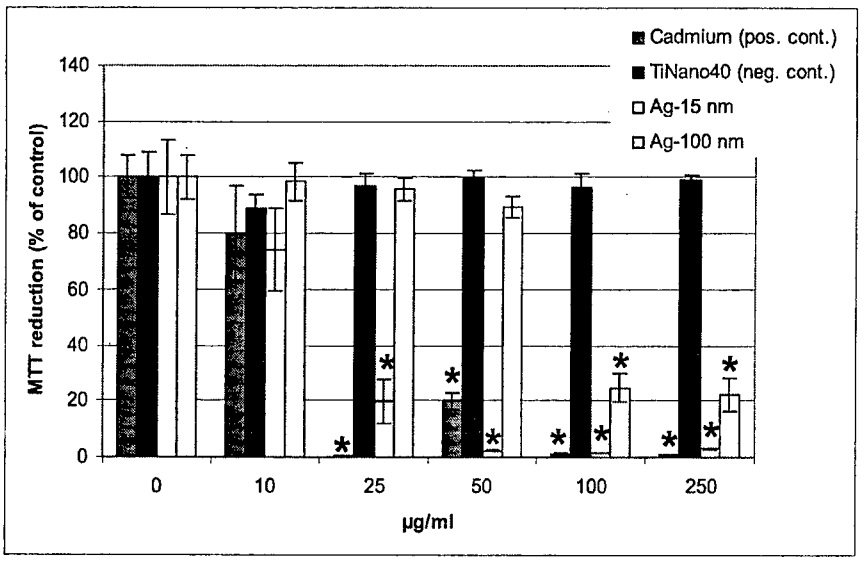

Figure 7: Effects of nanoparticles (24 hr exposure) on MTT reduction in bEnd.3 mouse endothelial cell line. *Significantly different from control (0 µg/ml).

At 50 µg/ml of Ag-15 nm, there was about a 70% reduction in mitochondrial function (Figure 6). As assessed by MTT assay, silver-15 nm nanoparticles were more toxic than Al and MoO_3 nanoparticles (Figure 5-6). However, there was a difference in the MTT assay results between the two cell types exposed to silver that indicates Ag-15 nm is more toxic in endothelial cells (Figures 6 and 7).

In addition, the Ag-15 nm is more toxic than the Ag-100 nm (Figure 6) in the endothelial cells where both silver particles were tested. A possible explanation for this difference could be the non-coated surface of the Ag-100 nm particles versus the carbon coating of the Ag-15 nm particles. The non-coated surface of Ag-100 nm made it difficult to disperse these particles into a homogenous suspension and may also have prevented dissolution of the Ag or the same degree of interaction of the particles with cells. The non-coated surface may have contributed to the lesser toxicity by providing a disproportionate exposure. The size difference between the 15 nm and 100 nm particles can not be compared due to the difference in the coatings between these two nanoparticles.

Neutral red is a water soluble, weakly basic, supra-vital dye that accumulates in lysosomes of viable cells. Cellular uptake of NR is accomplished either by pinocytosis or by passive transport across the plasma membrane. Accumulation of NR within lysosomes occurs

either from the trapping of the protonated form of the dye within the acid milieu of the lysosome or from the binding of NR to fixed acidic charges. In damaged cells, NR is no longer retained in cytoplasmic vacuolar membranes, and thus the dye is not retainable within the cell. The neutral red assay was chosen for this study to indicate whether cell viability during an exposure was compromised due to cytoplasmic membrane degradation. As assessed by NR assay, Ag-15 nm exposure exhibited a significant cytotoxicity from 25 – 250 μg/ml (Figure 8). At 100 μg/ml there was approximately a 60% reduction in membrane function in cells exposed to Ag-15 nm. Compared to Al, Ag was significantly more toxic when assessed by NR (Figure 8). The toxicity assessed by NR uptake assay followed the same pattern as assessed by the MTT assay in bEnd.3 cells.

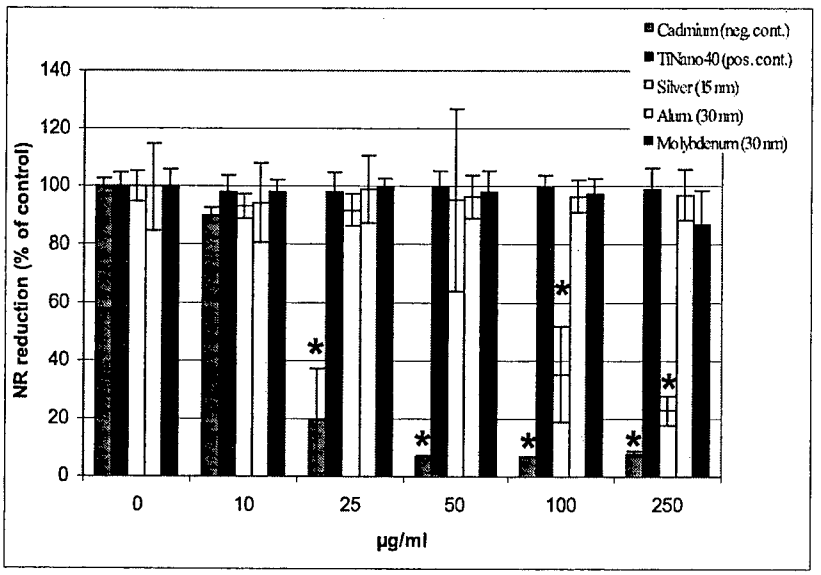

Figure 8: Effects of nanoparticles (24 hr exposure) on NR Reduction in HEL-30 mouse keratinocyte cell line. *Significantly different from control (0 μg/ml).

General external morphology of control cells and nanoparticle-exposed cells are demonstrated in Figures 9 through 12. The cells exposed to silver show a drastic change at higher concentrations. At the low dose (10 μg/ml), the cells appear similar to control cells. With increasing doses of Ag nanoparticles, the HEL-30 and bEnd.3 cells start to shrink and become irregular in shape; these changes are visible at 50 μg/ml. Microscopic studies indicate that

cytotoxicity is due to a chemical interaction with the nanoparticle, rather than a "smothering" effect due to high concentrations of nanoparticles per cell over the surface area.

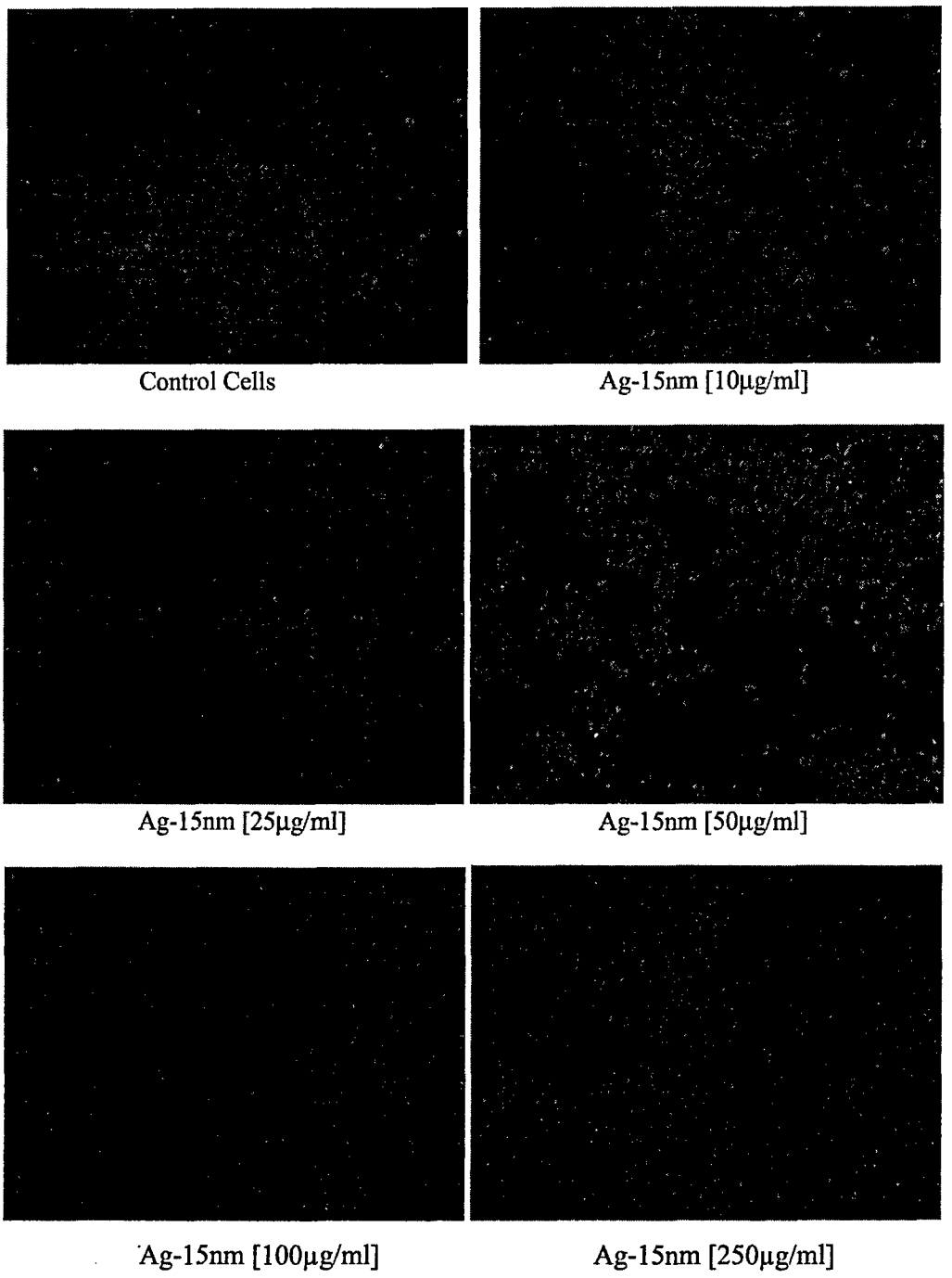

Figure 9: HEL-30 cells exposed to increasing Ag-15 nm concentrations

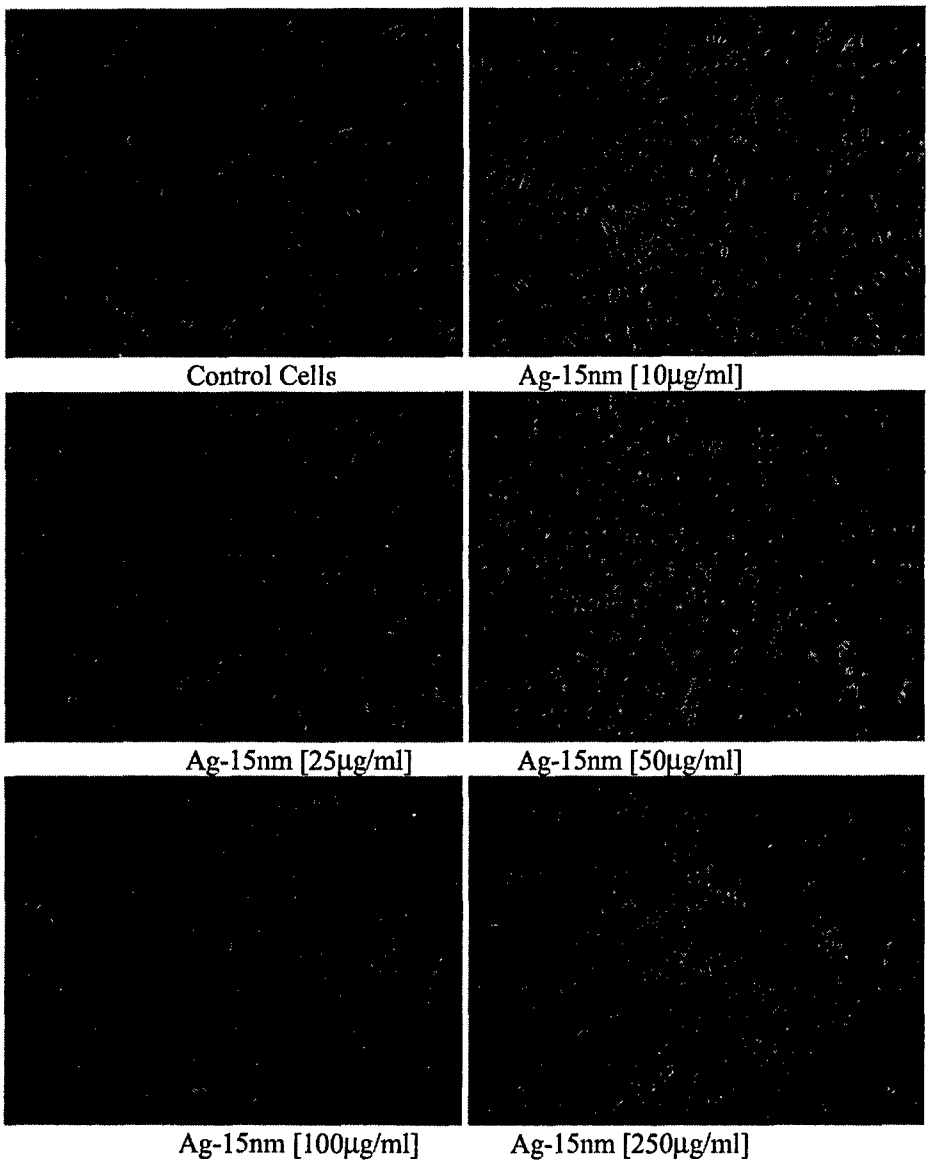

Figure 10: bEnd.3 cells exposed to increasing Ag-15 nm concentrations

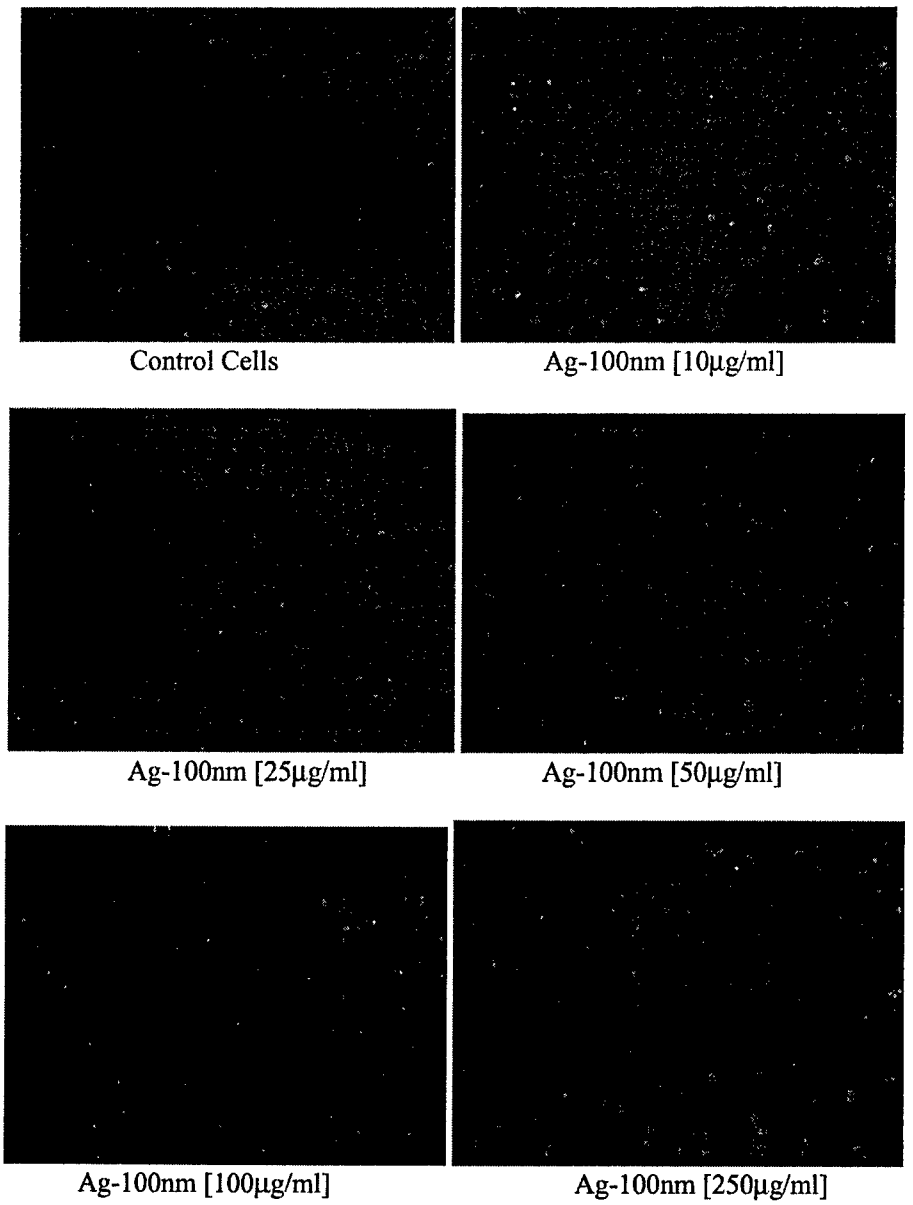

Figure 11: HEL-30 cells exposed to increasing Ag-100 nm concentrations

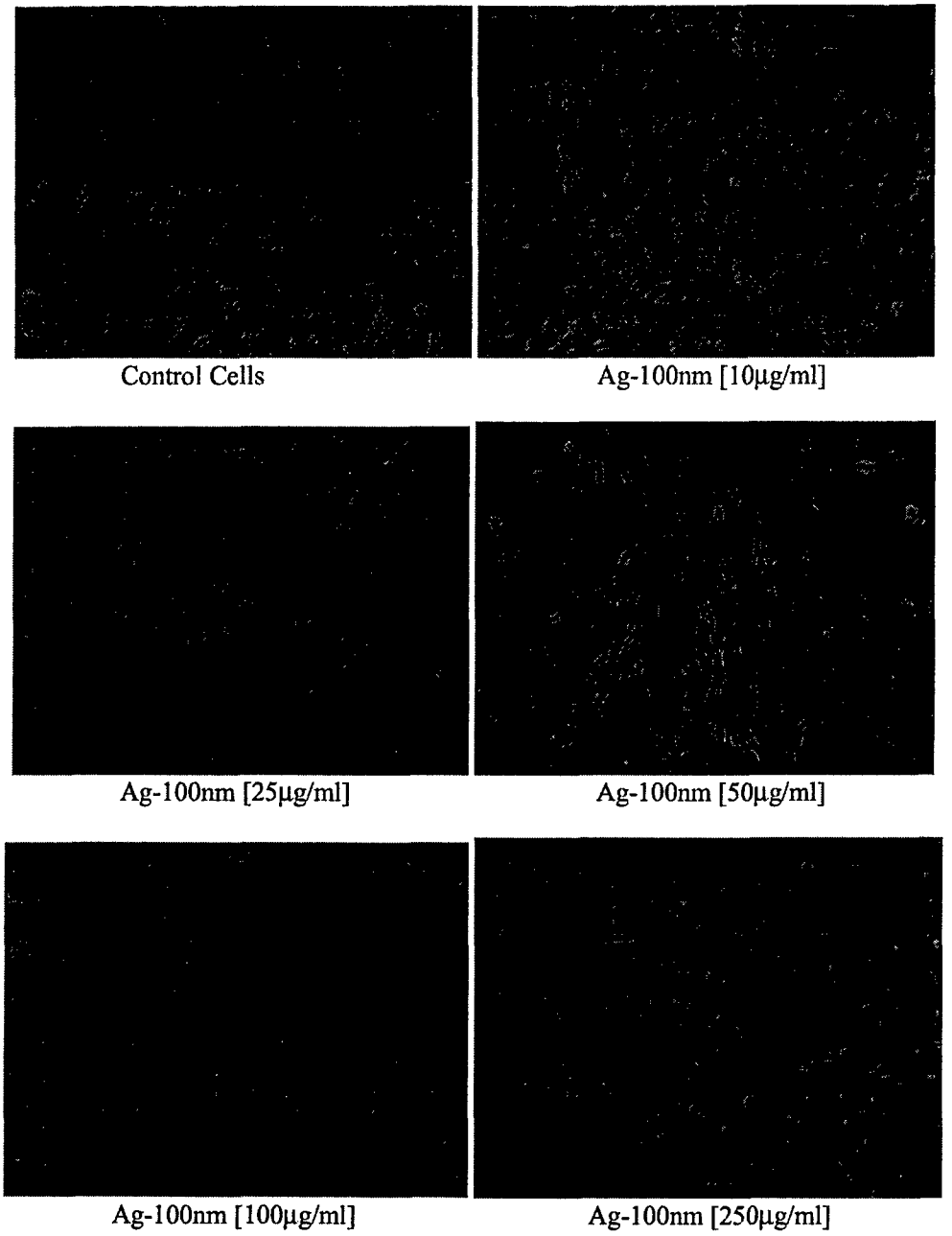

Figure 12: bEnd.3 cells exposed to increasing Ag-100 nm concentrations

3.3. Validation Results with Reference Chemicals:

In order to validate the assay system, results of nanoparticles were compared with other known positive and negative controls such as cadmium oxide (CdO) and titanium dioxide (TiO_2). The EC_{50} values of these compounds in the MTT assay for both cell lines are shown in Tables 1 and 2. The negative control, a titanium 40 nm particle, did not exhibit toxicity up to 250 µg/ml (Figures 5-8, as determined by MTT reduction in both the HEL-30 and bEnd.3 cell lines and the NR assay in HEL-30 cells). However, the positive control, CdO, displayed dose dependent toxicity with significant cell death at concentrations as low as 10 µg/ml, as determined by the NR assay in HEL-30 cells (Figure 8).

Table 1: EC_{50} values for nanoparticles in HEL-30 cells

Nanoparticle	Ec_{50} (µg/ml)	SD (± µg/ml)
[1]CdO	1.63	0.03
[2]TiO_2	>250	NA
Ag-15 nm	35.4	7.6
Ag-100 nm	>250	NA
Al	>250	NA
MoO_3	>250	NA

Note: [1]Positive Control, [2]Negative Control, SD = standard deviation

Table 2: EC_{50} values for nanoparticles in bEnd.3 cells

Nanoparticle	Ec_{50} (µg/ml)	SD (± µg/ml)
[1]CdO	1.63	0.03
[2]TiO_2	>250	NA
Ag-15nm	10.8	2.5
Ag-100nm	100.0	10.8
Al	>250	NA
MoO_3	>250	NA

Note: [1]Positive Control, [2]Negative Control, SD = standard deviation

3.4. Conclusions:

Based on our preliminary toxicity assessment, Ag nanoparticles are more toxic than Al and MoO_3 in keratinocytes. These results provide an early indication of potential toxicity concern of silver nanoparticles and may require further assessment to determine their biological interaction and potential, subsequent, adverse effects. Our earlier studies with Ag nanoparticles indicated that the toxicity of Ag-15 and Ag-100 nm nanoparticles involved oxidative stress (Hussain *et al.*, 2004, 2005). However, this study indicates that Ag-15 nm under our current experimental conditions may cause cell death and membrane damage, as well as obvious morphological changes. Further study is required to confirm the mechanism of toxicity of Ag-15 nm and other nanoparticles in keratinocytes. In addition, further investigation is required before extrapolating these results to whole animal systems.

4.0 – REFERENCES

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