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A Current Overview of the Molecular Effects of Nanosilver

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Abstract

Nanosilver plays an important role in nanoscience and nanotechnology, and is becoming increasingly used for applications in nanomedicine. Nanosilver ranges in size from one to one hundred nanometers. Smaller particles more readily enter cells and interact with the cellular components. The exposure dose, particle size, coating, and aggregation state of the nanosilver, as well as the cell type or organism that it is tested on, all have a large determining factor on the effect and potential toxicity of nanosilver. A high exposure dose to nanosilver alters the cellular stress responses and initiates cascades of signaling that can eventually trigger organelle autophagy and apoptosis. This review summarizes the current knowledge of the effects of nanosilver on cellular metabolic function and response to stress. Both the causative effects of nanosilver on oxidative stress, endoplasmic reticulum stress, and hypoxic stress, as well as the effects of nanosilver on the responses to such stresses, are outlined. The interactions and effects of nanosilver on cellular uptake, oxidative stress (reactive oxygen species), inflammation, hypoxic response, mitochondrial function, endoplasmic reticulum function and the unfolded protein response, autophagy and

apoptosis, angiogenesis, epigenetics, genotoxicity, and cancer development and tumorigenesis, as well as other pathway alterations are examined in this review.

Keywords: silver nanoparticles; nanosilver; endocytosis; oxidative stress; reactive oxygen species; inflammation; wound healing; hypoxia; mitochondria; endoplasmic reticulum stress; unfolded protein response; autophagy; apoptosis; angiogenesis; epigenetics; genotoxicity; cancer; anti-cancer

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Outline

Introduction

Nanosilver is extremely small particles of silver, with lengths of 1-100 nm in at least one dimension [1]. Commercially, it is often named colloidal silver or silver nanoparticles. Nanosilver is well recognized to have antimicrobial, antifungal, and antiviral properties, and for these reasons it is widely used today in many consumer products such as food packaging, sports clothing, electronics, cosmetics, medical devices, and bandages [2–6]. Nanosilver is used as an effective treatment against both gram-negative and gram-positive bacteria [7,8], as well as against human immunodeficiency virus (HIV) [9,10]. The number of applications of nanosilver, and its current usage in commercial products, means that unregulated environmental contamination by nanosilver and human exposure to nanosilver is now a reality. A cause for concern is that nanosilver surface oxidation releases Ag^+ ions which are known to be toxic [1]. In fact, the smaller the nanosilver

particle, the higher the surface area to volume ratio, and the more Ag^+ ions are released. The antimicrobial properties of nanosilver are due to this release of Ag^+ ions [5] as well as due to nanosilver specific effects such as inhibition of transcription by RNA polymerase [11]. The release of Ag^+ ions inside cells from nanosilver that entered in its nanoparticle form has been sometimes referred to as the "Trojan horse" effect [11–14]. However, the effects due to the released Ag^+ ions versus the effects due to the non-ionic nanosilver particle in cells is still under investigation, and varies depending on the experimental conditions [1]. Manshian *et al.* [15] measured the amount of Ag^+ ions released from nanosilver solutions by inductively coupled plasma mass spectrometry (ICP-MS), treated primary human umbilical vein endothelial cells (HUVEC) and murine C17.2 neural progenitor cells with silver nitrate ($AgNO_3$) at the appropriate concentrations corresponding to the nanosilver treatments, and found only slight toxicity directly from the Ag^+ ions. Lin *et al.* (2014) [16] used a filter assay and ICP-MS to determine the amount of Ag^+ ions released from a solution of PVP-coated nanosilver after 24hr, and found that level of Ag^+ ions to have no appreciable effect on the cells. The release of Ag^+ ions from polyvinylpyrrolidone (PVP) coated nanosilver (25 nm) was found to be 9.3% in cell culture media, with this concentration of $AgNO_3$ not decreasing the viability of human embryonic kidney (HEK 293T) cells [12]. A release of Ag^+ ions of 17.9% and 10.3% has also been reported in nanosilver treated mouse erythroleukemia (MEL) and human liver carcinoma (HepG2) cells respectively [11]. In immortalized murine bone-marrow derived pro-B (Ba/F3) cells, 21.6% of the nanosilver was released as Ag^+ ions, while no Ag^+ ions were detected in either deionized water or cell culture media with the same concentration of nanosilver [17]. Silver ion selective electrodes have also been used to measure the amount of Ag^+ ions released from nanosilver [11,18]. These studies indicate that the nanosilver itself and not just the released Ag^+ ions affect the cellular processes. In addition, silver may be taken up and

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localized in the cell differently depending on whether it is in its nanoparticle or ionic form, resulting in specific nanoparticle effects. Various factors can affect the quantity of Ag^+ ions released such as the size of the nanosilver, the pH of the environment it is in, the surface coating, or the formation of a protein cornea around the nanosilver [15,19–21]. In cells, nanosilver undergoes transformation from the elemental silver (Ag^0), to Ag^+ ions, to silver oxide species (Ag–O–), and finally to silver sulfide species (Ag–S–) upon binding to thiols [22]. In the environment, nanosilver undergoes sulfidation reactions to Ag_2S, greatly reducing its potential toxicity [23–25]. Lesser amounts of nanosilver are transformed into organic sulfide thiol complexes, $AgNO_3$, Ag-lactate, silver chloride (AgCl), or Ag_2O, with only minor amounts being released as free aqueous Ag^+ ions [26–28].

Nanosilver can be absorbed through inhalation (e.g., shoe sprays or during industrial manufacturing), oral ingestion (e.g., from food packaging or taken medicinally), skin contact (e.g., from bandages, cosmetics, or clothing), and injection (e.g., medicinal) [3,21,29]. Once in the body, nanosilver is transported to the liver via the portal vein. No nanosilver metabolites are known to be produced by the enzymes in the liver. The nanosilver is released into the blood stream where it can then bind to blood plasma proteins and blood cells and be distributed to all the organs in the body [29]. Nanosilver is able to cross the blood brain barrier as well as the placental barrier [3,30,31]. A 28 day oral exposure study by Van der Zande *et al.* [32] of male Sprague-Dawley rats to nanosilver (>20 nm) indicated that the highest levels of nanosilver were found in the liver and spleen, with lower levels being found in the testis, kidney, brain, and lungs. Inhaled nanosilver may also reach the brain through the olfactory bulb [33]. A gender-specific difference in nanosilver accumulation was seen in a 90 day oral exposure study with ~60nm nanosilver, where it was found

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that female Fischer 344 rats accumulated twice the amount of silver in their kidneys as did the male rats [34–36]. In terms of intracellular distribution, a related study examining the effects of AgCl fed to albino rats over one month resulted in Ag^+ ions mainly in the mitochondria of the liver cells, while rats fed AgCl over six months resulted in Ag^+ ions mainly located in the cytosol of the liver cells [37]. Exposure to large amounts of nanosilver over a long period of time can result in a condition known as argyria, where silver is deposited in the skin microvessels; or argyrosis, where silver is deposited in the eyes, causing a permanent bluish discolouration [1,38,39]. Excretion of nanosilver mainly occurs through the feces (>99%), with trace amounts of the nanosilver being excreted in the urine [32].

The physical characteristics of nanosilver such as size, shape, coating, and aggregation state are very important in its interactions and effects on living organisms [21]. Larger sized nanosilver (100 nm) may not enter the cell, and instead may exert indirect receptor-mediated signalling effects such as through serine/threonine protein kinase (PAK), mitogen-activated protein kinase (MAPK), and protein phosphatase 2A (PP2A) [40]. Smaller nanosilver particles can enter the cells, release Ag^+ ions, interact with the various biomolecules, and may bind to sulfur containing proteins such as glutathione (GSH), thioredoxin (TXN), thioredoxin peroxidase and superoxide dismutase (SOD) through their sulfhydryl groups [1,5,21,40].

The size of the nanoparticles reported in this review are the size of the metallic core, generally as indicated by transmission electron microscopy (TEM) imaging in the studies, or failing that, the size specified by the supplier. This size does not include the surface coating or hydration shell around the nanosilver particle. Additionally, nanosilver is sometimes found to aggregate in the cell

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culture media depending on the surface coating of the nanosilver used or the handling method [41], making the size even larger. PVP is one of the common nanosilver coatings used in experiments to stabilize the nanosilver and prevent aggregation [42]. The effects of PVP itself has been tested at the appropriate experimental concentrations on cells and found to not cause the effects that are observed when the cells are treated with PVP-coated nanosilver [42–44]. Citrate is another common stabilizer, and on its own did not decrease the life span of *Drosophila melanogaster* [18]. The dosage of the nanosilver is also very important in terms of the cellular effects and toxicity. Many studies use a high and toxic concentration in their experiments, however, lower non-toxic doses are more relevant to the actual environmental exposure levels [18]. An hormetic effect has been observed with lower doses triggering cell-survival pathways and somewhat protecting the cells against subsequent higher dose treatment which leads to cell death [21,45,46].

The use of controls in nanosilver studies is important for determining the cause of the observed effects. $AgNO_3$ is most commonly used as an Ag^+ ion control [47]; however, silver acetate ($C_2H_3AgO_2$) [48,49] or silver carbonate (Ag_2CO_3) have also been used [50]. If the Ag^+ ion control is used at the same concentration as the nanosilver treatment dose, the $AgNO_3$ will be much more toxic since there are many more silver ions present than in the nanosilver solution [18,51]. In order to treat cells with a relevant concentration of Ag^+ ions for the Ag^+ ion control: (1) ICP-MS may be performed on the nanosilver solution to determine the concentration of Ag^+ ions that are released [11,15,16,51], (2) viability assays may be done to determine the treatment concentrations for both the Ag^+ ion control and nanosilver that gives the same % cell viability [52], or (3) the nanosilver particles can be incubated in media for an experimentally relevant time, removed by centrifugation, and the cells then treated with the remaining media containing any released Ag^+ ions [40,53]. A

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nanoparticle control such as Cerium (Ce) nanoparticles [16,47] or polystyrene nanoparticles [50] may also be used, although this control is less common in nanosilver studies.

Uptake of Nanosilver

Nanosilver is mainly seen to be taken up into cells through endocytosis into vesicles, although diffusion of the nanosilver across the cell membrane into the cytoplasm may also occur [51,54–57]. It has been suggested that nanosilver may be able to diffuse across the membrane through induced lipid peroxidation and disruption of the plasma membrane [21,58]. As well, nanosilver and Ag^+ ions have been known to interact with copper transport channels and may be taken up through these [18,21,59]. In endocytosis, the material is taken up into early endosomes formed from the cell membrane. These develop into late endosomes and then into lysosomes, which have a lower internal pH [49]. The acidic environment in the lysosomes increases the release of Ag^+ ions from the nanosilver [21]. In a DNA microarray study, non-toxic treatment of HepG2 cells with 20 nm citrate-coated nanosilver for 4 hrs increased the expression of genes for both clathrin-dependent and clathrin-independent endocytosis [55]. Human mesenchymal stem cells (hMSC) treated with non-toxic levels of 50 nm PVP-coated nanosilver took up the nanosilver through clathrin-dependent endocytosis and macropinocytosis. Nanosilver aggregates accumulated inside the hMSC cells in late endosomes or lysosomes, but were not seen in the nucleus, ER, or Golgi apparatus using fluorescence and light microscopy [49]. Similar results were found with normal human lung fibroblast (IMR-90) cells treated with 6-20 nm nanosilver, where the nanosilver was taken up through clathrin-dependent endocytosis and micropinocytosis into endosomes and the nucleus, and then removed through exocytosis [60]. TEM images showed 10, 50, and 100 nm PVP-coated nanosilver contained within single or double membrane vesicles in HepG2 cells, with

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some of the 100 nm particles breaking down into smaller particles. Energy Dispersive X-Ray Spectroscopy (EDS) further confirmed that silver was in the vesicles [61]. TEM images of human acute monocytic leukemia cells (THP-1) treated with cytotoxic levels of 20 nm nanosilver showed that the nanosilver was contained in endosomes or lysosomes within the cells, but not in the nucleus or mitochondria. Nanosilver contained in these vesicles was then removed from the cells through exocytosis [22]. Similarly, in Chinese Hamster Ovary subclone K1 (CHO-K1) cells treated with 15.9 nm bovine serum albumin (BSA) coated nanosilver, TEM imaging showed nanosilver inside endosomes or lysosomes but not in the nucleus or mitochondria [62]. Similar results were also seen in NIH 3T3 mouse embryonic fibroblast cells, where nanosilver was observed to be contained in endosomes but not inside the nucleus, mitochondria, or golgi apparatus [63]. Nanosilver aggregates were observed in endosomes in U251 glioblastoma cells treated with citrate-coated nanosilver (15.26 nm) at a non-toxic concentration [64]. *In vivo*, examination of the livers of male Sprague Dawley rats treated with nanosilver (PVP-coated, 22.32 nm) via intraperitoneal injection revealed that the nanosilver was deposited in endosomes and lysosomes in Kupffer cells, and was generally in the liver cells closest to the blood vessels [65].

Other studies do report the presence of nanosilver inside the mitochondria and nucleus. Nanosilver was seen inside the mitochondria in human blood monocytes treated with 28 nm nanosilver [56]. Regarding the presence of nanosilver in the nucleus, nanosilver aggregates were observed to be free in the cytoplasm, contained in vesicles, and in the nucleus in hMSC cells treated with 46 nm nanosilver [54]. Nanosilver was taken up into the cytoplasm and nucleus in male somatic Leydig (TM3) cells and male somatic Sertoli (TM4) cells treated with 10 nm nanosilver at the concentration that results in 50% cell death, that is, the effective concentration that results in 50%

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of the maximal effect (EC_{50}) [66]. Finally, ICP-MS indicated the presence of silver in the cytosol and nucleus, with only a low amount of silver being detected in the membrane fraction in MEL cells treated with 25 nm PVP-coated nanosilver [11].

The amount of nanosilver internalized by cells depends on the size, shape, surface coating, and surface charge of the nanosilver [47,67]. Smaller sized nanosilver has been repeatedly reported to elicit greater cellular effects than larger nanosilver particles due to both increased uptake and increased intracellular interactions. HepG2 cells treated with 10 and 75 nm, PVP and citrate-coated nanosilver, internalized the most nanosilver when treated with the citrate-coated nanosilver, followed by the PVP-coated nanosilver, and took up the least when treated with silver nitrate. The smaller 10 nm nanosilver elicited a stronger oxidative stress pathway response than the larger 75 nm nanosilver [47]. In rat N27 neuronal cells treated with non-toxic levels of 10 and 75 nm, PVP and citrate coated nanosilver, non-toxic treatment with the PVP-coated nanosilver affected gene expression more than the citrate-coated nanosilver. Additionally, the smaller 10 nm PVP-coated nanosilver elicited stronger nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor and antioxidant response element (ARE) related gene activation, while than the larger 75 nm PVP-coated nanosilver activated more genes related to mitochondrial dysfunction, DNA damage, and kidney damage [67]. Similarly, smaller sized 10 and 25 nm PVP-coated nanosilver inhibited globin mRNA expression in MEL cells more than larger 40 and 110 nm nanosilver, and spherical nanosilver had a stronger effect than plate-like nanosilver [11].

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Cell type and function is also a large determining factor for nanosilver absorption. For example, phagocytic mouse BV2 microglia cells visibly took up nanosilver of all the sizes and coatings tested, while non-phagocytic N27 neuronal cells took up very little nanosilver [67].

Nanosilver and Oxidative Stress (Reactive Oxygen Species)

Reactive oxygen species (ROS) includes any form of oxygen with an unpaired electron in its outer electron orbital, such as superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical (•OH), singlet oxygen ($^1O_2$), alkoxy radicals (RO•), peroxy radicals (ROO•), hydochlorous acid, hypobromous acid, and others [68]. Superoxide dismutase (SOD) converts $\bullet O_2^-$ to oxygen (O_2) and H_2O_2, and H_2O_2 is then converted to water (H_2O) and O_2 by catalase (CAT). Low amounts of ROS are important signalling molecules; however, large amounts can cause cellular damage such as oxidation of proteins and DNA [60,69]. ROS are mainly produced in the mitochondria in cells. During oxidative phosphorylation, some electrons may escape and bind to O_2 forming $\bullet O_2^-$, which may be converted to other ROS such as H_2O_2 and •OH. Inhibition of oxidative phosphorylation may result in increased formation of $\bullet O_2^-$, as well as decreased ATP production [60]. Malondialdehyde (MDA) is produced as a result of lipid peroxidation and increased MDA is an indication of oxidative stress [70,71]. GSH is the major antioxidant molecule produced in cells [72]. Intracellular ROS depletes the levels of GSH, activates the cellular antioxidant response, causes DNA and mitochondrial damage, and ultimately leads to cell death if the cell cannot respond sufficiently [1,73,74]. Genes involved in the cellular oxidative stress response are up-regulated, such as heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide phosphate oxidase (NADPH)-quinone oxidoreductase (NQO1), Nrf2, TXN, NADPH oxidase 4 (NOX4), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit

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(GCLM), and metallothionein (MT), among others [46,55,67,75–79]. It has been generally thought that cellular nanosilver toxicity is mainly due to the production of ROS in the cell, leading to the depletion of cellular antioxidants such as GSH, the activation of the cellular antioxidant response, DNA and mitochondrial damage, and apoptosis [1,17,73,74]. However, conflicting results are reported in the literature regarding the production of ROS as a result of nanosilver treatment, with some reporting an increase in ROS, some reporting no change in ROS, and some reporting a decrease in ROS. Many different factors may be contributing to these results, including the methods used for detecting ROS, the exact ROS being measured, the relative sensitivities and unique responses of the various cells lines, and the nanosilver coating, size, dose, and treatment time that is used.

The majority of studies examining the effect of nanosilver on intracellular ROS production use 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and observe an increase in ROS [15]. This non-polar dye is thought to enter the cells via diffusion, where it is hydrolysed by esterase enzymes producing 2',7'-dichlorodihydrofluorescein (H_2DCF). Intracellular ROS in the form of peroxides oxidizes H_2DCF producing, 2',7'-dichlorofluorescein (DCF) which is strongly fluorescent and can be detected. However, there is confusion regarding the reliability of the assay, the proposed mechanism, which ROS are actually measured, and how much of the result is actually due to artifacts [63,66,68]. Additionally, the cells, media, and order of treatment used in doing the H_2DCFDA assay can affect the outcome [80]. A more reliable method is to use a fluorescent dye and flow cytometry with proper gating to remove artifacts due to background fluorescence and cell debris [81]. The CellROX fluorescent probes detect $\bullet O_2^-$ and •OH, but do not detect H_2O_2 well. CellROX Green Reagent detects ROS mainly in the nucleus and mitochondria, while CellROX

Deep Red and CellROX Orange detects ROS mainly in the cytoplasm. Resorufin is a dye specific for H_2O_2 [82], and dihydroethidium (DHE) is oxidized by $\bullet O_2^-$ [60]. Additionally, mitoSOX Red may be used to detect mitochondrial $\bullet O_2^-$ specifically [83].

Increase in ROS with Nanosilver Treatment:

A significant increase in ROS was seen with both non-toxic and toxic treatment of human embryonic stem cell-derived neural stem/progenitor cells (hESC-derived NPCs) when treated with 13.3 nm citrate-coated nanosilver and measured with the 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) dye [3]. Human chronic myeloid leukemia K562 cells exposed to non-toxic treatments of 27 nm PVP-coated nanosilver had a 30-40% increase in intracellular ROS as measured by H_2DCFDA, increased SOD activity, and a decrease in CAT activity [73]. ROS increased as measured by H_2DCFDA in NIH 3T3 cells treated with 26.2 nm nanosilver. Additionally, nanosilver treatment resulted in the depletion of intracellular GSH levels as well as an increase in HO-1 gene expression [63]. Nanosilver (10 and 20 nm) treatment of TM3 and TM4 cells at the IC50 resulted in a significant increase in ROS as measured with H_2DCFDA, and this increase was somewhat rescued using the antioxidant N-acetylcysteine (NAC). A decrease in mitochondrial activity and ATP production was observed alongside an increase in ROS as measured with H_2DCFDA and DHE in nanosilver (starch-coated, 6-20 nm) treated U251 and IMR-90 cells [60]. One of the downstream effects of the induced oxidative stress resulting from the nanosilver treatment was increased cellular damage and membrane leakage as determined with the lactate dehydrogenase (LDH) assay [66]. ROS measured with H_2DCFDA increased in MCF-7 breast adenocarcinoma cells with non-toxic nanosilver (10-30 nm) treatment below the EC_{50} [84]. ROS was also increased in human renal

proximal tubular epithelial (HK-2) cells treated with 7.5 nm nanosilver as measured with carboxy-H_2DCFDA, with the nanosilver treatment conditions resulting in approximately 60% cell viability [77]. Human lung carcinoma (A549) cells treated with 10 nm nanosilver at the EC_{50} increased the amount of ROS measured by H_2DCFDA fluorescence, however, hypoxia pre-treatment reduced the amount of oxygen present and attenuated this increase in ROS [85]. Nanosilver (18 nm) treatment at the EC25 increased the levels of ROS in human ovarian cancer (A2780) cells as measured with H_2DCFDA. Cytotoxicity, indicated by LDH release, also increased with this treatment. The level of MDA increased, and the levels of GSH, SOD, and CAT significantly decreased in cells treated with nanosilver at the EC25 concentration, indicating the induction of oxidative stress [71]. Similar effects were produced in A2780 cells by 20 nm nanosilver at the EC_{50}, and a nanocomposite made from 20 nm nanosilver dispersed on graphene sheets at the EC_{50} elicited an even stronger response [86]. GSH depletion was observed in HepG2 cells treated with 10 and 75 nm, citrate and PVP-coated nanosilver. The 10 nm citrate coated nanosilver resulted in the highest GSH depletion, followed by the 10 nm PVP-coated nanosilver, the 75 nm citrate-coated nanosilver, and finally the 75 nm PVP-coated nanosilver [47]. ROS increased in K562 cells with non-toxic nanosilver (27 nm, PVP-coated) treatment as measured with H_2DCFDA. The activities of the antioxidant enzymes SOD and CAT were measured, and the activity of SOD increased while the activity of CAT decreased [73]. In N27 neurons, the amount of GSH increased with increasing nanosilver treatment with 10 and 75 nm PVP-coated nanosilver and 75 nm citrate-coated nanosilver. Additionally, as another test for oxidative stress, the level of nitrous oxide was examined through measuring the levels of its metabolite, nitrite, in the cells; and increased nitrite levels were found in the N27 cells treated with 10 and 75 nm PVP-coated nanosilver [67]. Manshian *et al.* [15] measured ROS using CellROX green, and reports only a slight increase in

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ROS resulting from high treatment concentrations of nanosilver for both C17.2 cells and HUVEC cells treated with 4.2 nm mercaptoundecanoic acid (MUA) or dodecylamine-modified poly(isobutylene-*alt*-maleic anhydride (PMA) coated nanosilver; with only the PMA-coated nanosilver resulting in a significant increase in ROS at the highest treatment concentration [15].

In vivo, the effect of prolonged nanosilver (PVP-coated, 20-30 nm) exposure was examined in male Sprague Dawley rats by Blanco *et al.* [69]. The mice were treated with 0, low (50 mg/kg/day), medium (100 mg/kg/day), and high (200 mg/kg/day) doses every day for 90 days by gavage administration, and the effects on their livers assessed. The activity of SOD and CAT increased until the highest dose where it deceased. Lipid peroxidation also increased, together indicating a hepatic response to increased ROS [69].

No Change or a Decrease in ROS with Nanosilver Treatment:

On the other hand, no change or a decrease in ROS production due to nanosilver exposure is observed in several other studies. No increase in ROS was observed using the H_2DCFDA dye in HepG2 and human colon cancer (Caco-2) cells, even at concentrations that caused significant cytotoxicity, DNA damage, and mitochondrial injury to the cells [87]. With non-toxic nanosilver treatment, MEL cells exposed to 25 nm PVP-coated nanosilver (8 µg/mL) did not lead to any increase in ROS production as measured with H_2DCFDA [11,19]. Similarly, in HEK 293T cells treated with non-toxic levels of PVP-coated 25 nm nanosilver, there was no significant increase in ROS as measured with H_2DCFDA [12]. Human gingival fibroblasts (HGFs) treated with 30 nm nanosilver dispersed in a solution of Chitlac decreased the production of ROS compared to the untreated cells as measured with CM-H_2DCFDA, a chloromethyl derivative of H_2DCFDA [88].

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Thus, the majority of the studies indicate an increase in ROS and oxidative stress as a result of nanosilver treatment. However, this has been questioned in some studies where no increase in ROS was seen. This may be due to different experimental conditions or due to problems detecting the actual levels ROS with H_2DCFDA, the main dye that is used to detect ROS.

Nanosilver and Inflammation

Inflammation is an immune response to stress or injury in which leucocyte cells infiltrate the damaged tissue and mount an immune defence in order to destroy the source and aid in the healing process. Acute and short term inflammation is beneficial; however, chronic inflammation may lead to damage and diseases such as arthritis and cancer [89,90]. Cytokines such as interleukin-6 (IL-6), IL-1β, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and transforming growth factor-β (TGF-β) are produced by the leucocyte cells and stimulate changes in the production of acute phase plasma proteins as well as many other biochemical and physiological changes. In acute inflammation, the leucocyte cells are mainly neutrophils, while in chronic inflammation macrophages and lymphocyte cells are recruited approximately 48-96 hrs after initiation to aid in destroying the inflammatory agent and promote healing [89,91]. The nuclear factor kappa B (NF-κB) pathway is involved in the cellular response to various stresses including oxidative stress, and is involved in the start of inflammation. Phosphorylation of I-kappaB kinases (IKK) in response to ROS leads to the release of NF-κB dimers from inhibitory I-κB proteins, allowing them to enter the nucleus and activate gene expression leading to an inflammatory cellular response [92]. Cyclooxygenase-2 (COX-2) is a pro-inflammatory mediator during acute inflammation and is induced by cytokines such as IL-1β and TNF-α [91,93]. The activator protein 1 (AP1) transcription

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factor family consists of 18 dimers made from Fos, Jun, Maf, or activating transcription factor (ATF) family proteins. Phosphorylation of AP1 regulates its activity. AP1 is activated in response to various stimulants such as inflammatory cytokines, cellular stress, infection, or UV radiation; and once activated, AP1 has a role in various cellular responses including inflammation, cell survival, differentiation, proliferation, and apoptosis [94].

Nanosilver and Inflammation in vitro:

The NF-κB and AP1 pathways were activated by nanosilver (10 and 75 nm, citrate and PVP-coated) in stable luciferase-reporter HepG2 cells [47] and in N27 neurons [67]. Non-toxic nanosilver (citrate-coated, 5 nm) treatment activated the NFκB pathway in human cervical cancer cells (HeLa) and A549 cells, and an increase in the cellular immune response was seen as an increase in the pro-inflammatory cytokine IL-1α [92]. The levels of IL-6 and IL-8 increased in hMSCs treated with non-toxic nanosilver (46 nm) treatment) [54]. A slight increase in prostaglandin E2 and a significant increase in IL-6 was observed in HGFs treated with 30 nm nanosilver dispersed in a solution of Chitlac, a biocompatible modified polysaccharide composed of a chitosan backbone to which lactitol moieties have been chemically inserted [88]. Additionally, non-toxic nanosilver treatment (PVP-coated, 10, 50, and 100 nm) resulted in activation of the NLRP3-inflammasome via activated caspase-1 and increased IL-1β secretion in HepG2 cells, with the 10 nm nanosilver being the most potent [61].

Nanosilver and Inflammation In Vivo:

Nanosilver treatment has been seen to activate the immune system and cause inflammation in mice. In a study by Park *et al*. [95], male and female ICR mice orally treated with nanosilver (22, 42, 71

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nm) at 1 mg/kg for 14 days resulted in an increase in TGF-β in the serum and increased distribution of B cells and natural killer cells, although the body weight of the mice did not change. Larger 323 nm silver particles did not cause any significant effect. Longer term treatment of the mice with 42 nm nanosilver at various doses (0.25, 0.5, and 1 mg/kg) for 28 days only showed adverse effects in the liver and kidney at the highest treatment dose, with increased levels of alkaline phosphatase, aspartate transaminase, and alanine transaminase; with the later only observed in female mice. An increase in the levels of TGF-β, IL-1, IL-4, IL-6, IL-10, IL-12, and immunoglobulin E (IgE) antibody was observed in the plasma at the higher treatment doses, and the B cell distribution also increased [95]. Nanosilver injected peritumorally around tumors made from murine lung squamous tumor cells (KLN 205) in female immune competent DBA/2 mice and immune deficient NOD SCIDγ mice resulted in inflammation in the immune competent DBA/2 mice as visualized with an inflammation-activatable probe (Cat B 750 FAST) and 3D optical imaging [92]. Inflammation has also been observed in the liver tissue of male Sprague Dawley rats treated with PVP-coated nanosilver (22.32 nm) by interperitoneal injection [65].

Anti-inflammatory Properties of Nanosilver in Wound Healing:

The process of skin wound healing involves inflammation, proliferation, and tissue remodeling. The injury stimulates inflammation and the release of pro-inflammatory cytokines. Granulation tissue formation and angiogenesis occurs during proliferation and is aided by the macrophages. During tissue remodeling, damaged tissue is removed and the extracellular matrix is remodelled, with this final process being controlled by various matrix metalloproteinases (MMPs) and tissue inhibitors [91]. Nanosilver treatment has been found in multiple studies to be beneficial in wound healing [96–100]. Inflammation is an integral part of wound healing; however, prolonged

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inflammation delays new cell proliferation and slows down healing, while shortened inflammation quickens the healing process [91,101]. In a skin wound healing model with normal human dermal fibroblasts (NHDFs) and normal human epidermal keratinocytes (NHEKs), nanosilver (10 nm) treatment decreased the expression of TNF-α, IL-12, COX-2, vascular endothelial growth factor (VEGF), and MMP-3, thus serving to speed up the healing process [91]. In a thermal injury animal model using male BALB/C mice, bandages coated with nanosilver (14 nm) decreased the inflammation compared to the control mice, eliminated bacterial growth, and resulted in faster healing with reduced scarring. Nanosilver treatment also affected the mRNA expression of various cytokines. IL-6 was down-regulated, while IL-10, VEGF, and IFN-γ were all up-regulated. TGF-β1 was initially up-regulated, and then was down-regulated later in the healing process [96]. One millilitre of various concentrations (9, 45 and 90 μM) of 9.3 nm nanosilver were put in a wound in BALB/C mice before it was surgically closed, and the highest concentration of nanosilver greatly decreased the severity of postoperative peritoneal adhesions as well as decreasing inflammation. In cell culture studies with the mouse macrophage cell lines RAW264.7 and J774.1, nanosilver treatment was able to decrease the levels of TNF-α produced as a result of lipopolysaccharide (LPS) induced inflammation [97]. Studies have been conducted combining nanosilver treatment with other compounds and natural extracts, which have resulted in even more effective wound healing and decrease in inflammation [98,101–103].

The results from the *in vitro* and *in vivo* studies are in agreement with the results from the wound healing studies. Since inflammation is an important step in the wound healing process, the increase in inflammation due to the nanosilver treatment reported in the *in vitro* and *in vivo* studies would

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lead to an increased speed of wound healing and a faster decrease in inflammation, which is seen in the wound healing studies.

Nanosilver and Hypoxia Stress

Low oxygen (hypoxic) conditions are encountered by humans during various physiological (high altitude), developmental (during embryogenesis) and clinical conditions, (during embryogenesis, cardiac arrest, stroke, and in solid tumors) [104]. The cellular response to hypoxia involves the activation of the transcription factor hypoxia-inducible factor (HIF). Under normal oxygen conditions (normoxia), the alpha subunit of HIF (HIFα) is hydroxylated by oxygen-dependent prolyl hydroxylase enzymes leading to the recognition of HIF-α by E3 ubiquitin ligases, its subsequent ubiquitination and proteasomal degradation. Additionally, hydroxylation of HIFα terminal transactivation domain by Factor Inhibiting HIF (FIH), an asparaginyl/aspartic acid hydroxylase, blocks the coactivator CREB-binding protein/p300 from binding to HIF-α, which is required for complete HIF-α activation. Under hypoxic conditions, HIFα hydroxylation and degradation is inhibited, and HIF-α translocates to the nucleus where it binds to HIF-β (also known as the aryl hydrocarbon receptor nuclear translocator (ARNT)). This heterodimer binds to the hypoxic response elements (HRE) in the promoters of HIF target genes, thus activating gene expression integral for adaptation to the hypoxic stress [104,105]. HIF target genes include genes involved in a) glucose metabolism and transport (glucose transporter type 1 and type 3 (GLUT1 and GLUT3)), b) glycolysis (hexokinases I and II (HKI and HKII), phosphofructokinase-L (PFK-L), aldolases-A and -C (ALD-A and ALD-C), phosphoglucokinase-1 (PGK1), enolase alpha (ENO-α), pyruvate kinase M2 (PYK-M2), lactate dehydrogenase-A (LDH-A) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB-3)), c) angiogenesis (vascular

endothelial growth factor-A (VEGF-A)), d) erythropoiesis (erythropoietin (EPO)), and e) iron transport and metabolism (hepcidin (HAMP), ferroportin (SLC40A1), transferrin receptor (TFR1), divalent metal transporter 1 (DMT1)) [57,104].

Only a few studies have focused on the effects of nanosilver treatment on HIF-1α expression, and on the effects of nanosilver treatment in combination with hypoxia treatment, with some conflicting results. In A549, HIF-1α protein expression was found to increase in hypoxia treatment, EC_{50} level nanosilver treatment, as well as showing additive effects with both the hypoxic and nanosilver treatments together [85]. Similarly, an increase in HIF-1α expression was observed in hMSCs with non-toxic nanosilver treatment of up to 7.5 μg/mL [106]. Gene expression studies with C17.2 cells indicated the upregulation of several HIF target genes: adrenomedullin, HO-1, and serpine1 with non-toxic nanosilver treatment [15]. The levels of VEGF increased with non-toxic treatment of 46 nm nanosilver on hMSCs [54]. As well, two studies on nematodes (*Caenorhabditis elegans*) also reported an increase in HIF-1α activation with nanosilver exposure [107,108]. However, the opposite result is reported by Yang *et al.* [57] who observed decreased protein expression of HIF-1α, VEGF-A, and GLUT1 in human breast cancer MCF-7 cells that were treated with a combination of hypoxia and non-toxic nanosilver treatment of 10 μg/mL, as compared to cells treated only with hypoxia. An even further decrease was observed when the cells were treated with a toxic concentration of nanosilver along with hypoxic treatment [57]. In support of these results, a study using female BALBc mice treated with ovalbumin inhalation to model allergic airway inflammation, found that nanosilver treatment reduced the effects of the ovalbumin treatment by lowering the expression of HIF-1α and VEGF [109]. Thus, more research needs to

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be done to understand the effects that nanosilver treatment has on the hypoxic response pathway and on HIF-1α expression.

Nanosilver and the Mitochondria

The mitochondria is the cellular site of ATP production, through oxidative phosphorylation, and this organelle is integral in cell signaling, cell proliferation, and cell death pathways [110]. The mitochondria forms a complex reticular network throughout the cytosol, allowing communication between it and other organelles within the cell, and undergoes fission or fusion in response to the cellular conditions [111]. The endoplasmic reticulum (ER) is an organelle that also forms a reticular network throughout the cytosol and is in close communication with the mitochondria for the purpose of the transfer of ions, proteins, and lipids. The areas where the mitochondria and ER membranes associate are known as mitochondria-associated membranes (MAMs) and these areas are involved in interorganelle communication through various receptors and channels [112]. ER stress is known to cause the release of calcium from the ER to the cytoplasm through inositol 1,4,5-triphosphate receptors (IP3R), and this released calcium can further stimulate calcium-induced calcium release (CICR) through the ryanodine receptors in the ER membrane [113,114]. This release of calcium from the ER subsequently leads to the uptake of calcium into the mitochondria. Additionally, the MAM protein, phosphatase and tensin homolog deleted on chromosome ten (PTEN), is thought to increase the release of calcium from the ER by decreasing the phosphorylation of IP3R, and thus increasing the transfer of calcium to the mitochondria [114]. In neurons, stimulation of the excitatory glutamate receptor: N-methyl-D-aspartate (NMDA) receptor, allows calcium to flow from the extracellular space into the neuron. This influx of calcium into the neuron stimulates CICR as well as IP3R-mediated calcium release from the ER

calcium reserves into the cytoplasm, leading to increased mitochondrial calcium levels and potentially to mitochondrial dysfunction [115,116]. Mitochondrial mediated apoptosis (intrinsic apoptosis) involves the release of cytochrome c, triggering a caspase signalling cascade. Caspase-8 and caspase-9 are initiator caspases that activate the executioner caspases, caspase-3 and caspase-7 [66]. The Bcl-2 family of proteins is also involved in mitochondrial mediated apoptosis; including the pro-apoptotic proteins Bax and Bak, and the anti-apoptotic protein Bcl-2 [71].

Detection of the intracellular calcium levels (via radioactive calcium and the calcium-sensitive fluorescent probe, fluo-3 AM) in primary rat cerebellar granule cells (CGC) treated with various combinations of NMDA receptor agonists, NMDA receptor antagonists, and nanosilver, indicated that nanosilver treatment increased intracellular calcium levels through NMDA receptor activation [116]. Nanosilver treatment of human Chang liver cells at the EC_{50} increased the mitochondrial calcium level in the cells by over two-fold, as was seen by flow cytometry with the Rhod2-AM fluorescent probe [117]. Rhod2-AM sequesters preferentially into the mitochondria due to its positive charge, as well as only fluorescing once it is oxidized which generally occurs in the mitochondria [117], thus making it useful in detecting mitochondrial calcium levels.

The length of membrane in close contact between the ER and the mitochondria was seen to visibly increase with nanosilver treatment of human neuroblastoma (SH-SY5Y) cells in TEM images. Additionally, ER- and mitochondrial-specific staining showed that the co-localization of the ER and mitochondria increased with nanosilver treatment [114]. PTEN moved from the cytoplasm to the ER and MAMs with nanosilver treatment. Co-immunoprecipitation of PTEN with IP3R

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indicated direct interaction between PTEN and IP3R, as well as showing a decrease in phosphorylated IP3R with toxic nanosilver treatment [114].

Mitochondria normally have a negative electric potential across the inner mitochondrial membrane, and disruption of mitochondrial homeostasis leads to depolarization and decrease in the membrane potential [87]. Mitochondrial membrane potential depolarization was seen via JC-1 mitochondrial staining once the nanosilver treatment reached the EC_{50} level for the SH-SY5Y cells. Along with this, decreased ATP production was seen, as well as increased Bax/Bcl-2 protein ratio, and increased protein levels of mitochondrial mediated apoptosis proteins: caspase-3, cleaved caspase-3, cytochrome c, and cleaved caspase-9 [114]. In A549 cells, the mitochondrial membrane potential as seen with JC-1 staining decreased when the cells were treated with 10 nm nanosilver at the EC_{50} level. As well, nanosilver treatment increased mitochondrial mediated apoptosis through an increase in activated caspase-3. Pre-exposure with hypoxia lessened the effects of the nanosilver treatment on the mitochondrial membrane potential and the mitochondrial damage [118]. A decrease in mitochondrial membrane potential was also seen with JC-1 staining in A2780 treated with nanosilver (18 nm) at the EC25 [71]. Mitochondrial mediated apoptosis was induced as seen by an increase in Bax and Bak mRNA, a decrease in Bcl-2 mRNA, an increase in caspase-9 and caspase-3 mRNA, and a decrease in pro-caspase-3 mRNA expression. Further, the use of a caspase-3 inhibitor successfully blocked the increased caspase-3 activity due to the nanosilver treatment [71]. Similarly, at high nanosilver treatment, caspase-7 and caspase-9 had increased processing and activity in MCF-7 cells [119]. However, lower nontoxic nanosilver treatment of human bronchial epithelial (16HBE) cells did not lead to mitochondrial mediated apoptosis since cleaved caspase-3 was not detected in these cells [120]. Reduced mitochondrial

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membrane potential was indicated by a decrease in retention of the positively charged rhodamine 123 (R123) fluorescent dye was observed in HepG2, Caco-2 cells [87], and CGC cells [116,121]. The HepG2 cells were more sensitive and responded to lower dose nanosilver treatment whereas the Caco-2 cells and CGC cells only showed a decrease in membrane potential at a dose that corresponded to the EC_{50} of the cells [87,116,121]. Another fluorescent cationic dye, MitoTracker Red, also showed a decrease in mitochondrial membrane potential with high nanosilver treatment in human colon carcinoma cells (HCT116) [122]. Mitochondria mediated apoptosis was triggered by high dose nanosilver treatment as was seen by increased levels of activate phosphorylated c-Jun N-terminal Kinase (JNK), the translocation of the pro-apoptotic protein Bax to the mitochondria, and cytochrome C release into the cytoplasm [122,123]. Visible mitochondrial damage was caused in TM3 and TM4 cells treated with 10 and 20 nm nanosilver at the EC_{50}, and mitochondrial mediated apoptosis was induced as seen with an increase in caspase-3, caspase-8, and caspase-9 mRNA expression [66]. As well, impaired energy metabolism was seen as a drastic decrease in ATP production in liver tissue from male Sprague Dawley rats injected intraperitoneally with nanosilver (PVP-coated, 22.32 nm). Apoptosis also increased with the nanosilver treatment, with an increase in cleaved caspase-3 as well as increased DNA fragmentation as seen with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay [65].

Thus, high dose nanosilver treatment around the EC_{50} value for the cells leads to increased contact and signaling between the ER and the mitochondria, increased transfer of calcium from the ER to the mitochondrial, mitochondrial dysfunction, decrease in ATP production, and mitochondria-mediated apoptosis.

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Nanosilver and ER Stress (Unfolded Protein Response)

The ER is the main site in the cell for protein synthesis and folding, as well as being vital for lipid biogenesis and calcium storage [114,124]. Various cellular stresses such as hypoxia, oxidative stress, low glucose levels, viral infection, pharmaceuticals, or environmental stressors can cause unfolded or misfolded proteins to build up in the ER, a situation that is described as ER stress [124–126]. ER stress will activate the unfolded protein response (UPR) signaling pathway to return the cell to its healthy state, or if this is not possible, to lead to cell death. Pro-survival mechanisms include reducing general protein synthesis, increasing the number of chaperones produced in order to alleviate the ER stress, and increasing protein degradation [127,128]. Protein kinase RNA-like ER Kinase (PERK), ATF-6, and Inositol-Requiring Enzyme-1α (IRE-1α) are three ER membrane spanning proteins that act as sensors for ER stress. They are inactive when bound to Grp78, an ER luminal chaperone that is also called BiP [119,125]. Under ER stress, Grp78 dissociates from these complexes to assist in protein folding, thus activating the three ER sensors and their respective UPR pathways [125].

Upon dissociation of both Grp78 and Hsp90 from PERK, homodimerization and autophosphorylation turns PERK into an active kinase that will in turn phosphorylate eukaryotic translation initiation factor 2 (eIF2-α). Phosphorylated eIF2-α inhibits protein translation, thus reducing the number of new proteins being synthesized and reducing some of the load on the ER [125,126]. If the ER stress persists and cannot be corrected, then cell death pathways are triggered. The PERK/eIF2-α pathway triggers apoptosis by increasing the translation of mRNAs for certain transcription factors such as ATF-4. ATF-4 regulates many genes integral to cell survival and the

cellular stress response, but under these conditions of prolonged ER stress, ATF-4 stimulates the translation of CCAAT/enhancer-binding protein-homologous protein (CHOP or GADD153), which initiates apoptosis and suppresses the transcription of anti-apoptotic Bcl-2 family proteins [117,119,129]. Apoptosis due to prolonged ER stress can also be stimulated through the release of calcium from the ER, activating calpain, caspase-12, and caspase-3 apoptotic signaling cascade, with caspase-12 being the executor for ER mediated apoptosis and caspase-3 being the executor for mitochondrial mediated apoptosis [114,130]. Dissociation of Grp78 from ATF-6 allows this transcription factor to be transported to the Golgi apparatus and activated by proteolytic cleavage by transmembrane Site-1 and Site-2 proteases. The active N-terminal fragment of ATF-6 can then stimulate the needed gene expression either for survival (such as ATF-4 and X-box binding protein 1, XBP1) or for cell death [119,126,128]. Dissociation of the chaperones Grp78 and Hsp90 from IRE-1α allows these kinase/endoribonuclease enzymes to become active through homodimerization and autophosphorylation. Active IRE-1α excises an intron from XBP1 mRNA allowing it to be translated into XBP1 protein, a transcription factor that induces the expression of various target genes such as Grp78 [117,125]. IRE-1α can also phosphorylate and activate JNK. Active JNK phosphorylates Bcl-2 family proteins suppressing apoptosis [119].

Recently within the last six years, novel research has been published on the effects of nanosilver on ER stress and the UPR. Treatment of human Chang liver cells with nanosilver ≤ 100nm at a high concentration corresponding to the EC_{50} value of the cells (4 μg/ml for 24hr), showed increased protein levels of released chaperone Grp78, phosphorylated PERK, phosphorylated eIF2-α, phosphorylated IRE-1, spliced XBP1, active cleaved ATF-6, CHOP, and caspase 12; all of these being indications of the UPR [117]. In addition to this, siRNA knockdown of PERK,

IRE1, ATF-6, and CHOP reduced the intensity of the ER stress resulting from nanosilver treatment [117]. Similar results were seen in SH-SY5Y cells with 31.1 nm PVP nanosilver treatments up to the EC_{50} value of the cells (12.5 μg/ml for 24 hr) [114]. Increased levels of the same proteins seen by Zhang *et al.* [117] in Chang liver cells were observed in the SH-SY5Y cells, in addition to an increased Bax/Bcl-2 ratio and increased cleaved caspase 3 for the apoptotic regulator proteins [114]. Increased mRNA levels were also seen for Grp78, XBP-1, and CHOP, further evidence of high nanosilver treatment inducing ER stress and the UPR [114]. Treatment of MCF-7 and T-47D breast cancer cells with high concentrations of 2 and 15 nm nanosilver increased the protein levels of phosphorylated PERK, phosphorylated eIF2-α, and phosphorylated IRE1α. Inhibitors of ER stress were able to attenuate the toxicity due to the high nanosilver treatments, indicating that the ER stress response pathways were contributing to the cell death [119].

At lower non-toxic nanosilver treatment concentrations, perturbations in ER homeostasis are observed but not cytotoxicity with 20 nm nanosilver [130]. PERK and Hsp70 protein levels were increased with low nanosilver treatment in THP-1 cells, while no difference in the protein levels of Grp78 and ATF-6 were observed. Interestingly, ATF-6 degradation and cell death in the THP-1 cells were seen only with very high nanosilver treatments of 15 nm nanosilver [126]. Non-toxic nanosilver treatment did increase the protein level of Grp78 in 16HBE cells, but not in HepG2 or HUVEC, indicating that 16HBE cells are more sensitive to ER stress response due to nanosilver [130]. The protein level of JNK increased in all three of these cell lines by non-toxic nanosilver treatment. Additionally, in 16HBE cells there was more caspase-12 expressed than caspase-3, and only cleaved caspase-12 was detected; thus indicating ER mediated apoptosis [130].

The mRNA levels of spliced XBP-1 and CHOP were up-regulated in 16HBE cells in response to low nanosilver treatment. HepG2 cells were found to be less responsive to nanosilver treatment in this study, and only CHOP mRNA was up-regulated, with no increase in spliced XBP-1. HUVEC cells were also examined and no change was seen in the mRNA expression of spliced XBP-1 or CHOP with nanosilver treatment [130]. Fluorescence imaging showed the induction of CHOP in HepG2 cells with non-toxic nanosilver treatments of 10, 50, and 100 nm PVP nanosilver [61].

A human UPR PCR array test with 16HBE cells indicated nine genes that were up-regulated by more than threefold by nontoxic nanosilver treatment. These included genes for heat shock proteins: *heat shock 70kDa protein 1 beta (HSPA1β), heat shock 105kDa/110kDa protein 1 (HSPH1), DnaJ (Hsp40) homolog subfamily B member 9 (DNAJB9)*, and ER stress markers: *CHOP, protein phosphatase 1 regulatory (inhibitor) subunit 15A (PPP1R15A), homocysteine inducible ER protein with ubiquitin-like domain 1 (HERPUD1), adrenomedullin 2 (ADM2), asparagine synthetase (ASNS), and pseudokinase tribbles homolog 3 (TRIB3)* [130]. The large number of chaperone proteins upregulated by nontoxic nanosilver treatment is further evidence that these doses initiate the unfolded protein response. Thus, low dose exposure to nanosilver can disrupt ER homeostasis, and cause the induction of the ER stress response without cell death. High dose exposure to nanosilver, with treatments around the EC_{50} value for the cells or higher, leads to an induction of the ER stress response and cell death.

Effects of Nanosilver on Autophagy and Apoptosis

Autophagy refers to the cellular-mediated degradation of proteins, sugars, lipids, and some organelles via the lysosome [131]. This degradation may be activated either to remove damaged

cellular components, or as a protective response to stress such as starvation, stroke, hypoxia, radiation, or chemotherapy [16,85]. Many different nanoparticles have also been found to induce autophagy [16,42]. Autophagy recycles cytosolic components to aid in cell survival, however, prolonged autophagy results in excessive degradation and leads to cell death [16]. There are three different autophagic pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy, with the term autophagy generally being used to refer to macroautophagy. In macroautophagy, the ER is stimulated to form an omegasome, which matures into an isolation membrane (also called a preautophagosome or phagophore), and subsequently elongates to form a structure with a double-membrane called an autophagosome which closes around the components to be degraded. The autophagosome fuses with lysosomes forming an autolysosome, and the contents as well as the inner autophagosome membrane are degraded by lysosomal hydrolases. Permease enzymes in the lysosome membrane release the degradation products back into the cytosol for further cellular use. Microautophagy involves the lysosome directly engulfing the components to be degraded without the formation of an autophagosome. Finally, in chaperon-mediated autophagy, misfolded or unwanted proteins containing the KFERQ motif are recognized by the heat shock cognate 71kDa protein (Hsc70) chaperone, brought into the lysosome by a lysosomal membrane protein, lysosome-associated protein-2A (LAMP2A), and the proteins subsequently degraded [131,132].

Transcription factor EB (TFEB) is integral to regulating autophagy and lysosomal related genes, such as microtubule associated protein 1 light chain 3 beta (MAPLC3B), sequestosome 1 (p62/SQSTM1), UV radiation resistance-associated gene (UVRAG), WD repeat domain phosphoinositide-interacting protein 1 (WIPI1), vacuolar protein sorting-associated protein 11

homolog (VPS11), vacuolar protein sorting-associated protein 18 homolog (VPS18), autophagy related 9B (*ATG9B*), and genes in the coordinated lysosomal expression and regulation (CLEAR) network respectively [44,133]. Active mammalian target of rapamycin complex 1 (mTORC1) phosphorylates TFEB, causing it to be inactive and located in the cytosol. Various stress conditions such as starvation or lysosomal stress will deactivate mTORC1, allowing TFEB to be dephosphorylated and to translocate to the nucleus and regulate gene expression. As well, extracellular signal-regulated kinases 1/2 (ERK1/2) and protein kinase C β (PKC β) are also involved in the phosphorylation of TFEB [44]. Many of the *ATG* genes are involved in autophagosome formation [131]. Several of the autophagy proteins are also involved in activating apoptosis, including ATG3, ATG5, ATG6/Beclin1, ATG7, ATG10, ATG12, and ATG17 [71,132]. HIF-1α is another gene essential to the autophagy process [85]. Microtubule-associated protein 1 light chain 3 (LC3) is associated with autophagosome formation, exists as either LC3-I or LC3-II, and is used as a marker for autophagy. LC3-I is a soluble protein found in the cytosol. Under starvation or other autophagy promoting conditions LC3-I is cleaved by autophagin and conjugated to phosphatidylethanolamine, converting it to LC3-II, which is then incorporated into the isolation membrane and autophagosome membrane. Generally, an increase in LC3-II indicates an increase in autophagosome accumulation, however, care must be taken in interpreting these results since LC3-II is degraded with the autolysosomes during normal turnover and immunoblotting problems may occur [17,134,135]. TEM imaging is an important method for visualizing autophagosomes [41]. Acidic vesicular organelles (AVOs) can be detected via acridine orange (AO) dye which accumulates in acidic vesicles [63,136,137]. Both new autophagosome formation and the blockage of autophagosome degradation and turnover will result in an increase in autophagosomes and LC3-II in the cells, and care must be taken in order to determine whether

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autophagy induction or the blockage of autophagic flux has occurred [16,138]. In order to determine this, the degradation of p62/SQSTM1 can be examined. p62 binds to LC3, is incorporated into autophagosomes, and is degraded during autophagy. However, change in p62 protein expression is not specific to autophagy and should not be used exclusively to show changes in autophagy [134]. Inhibition of autolysosomal degradation and turnover, such as with bafilomycin A1 or chloroquine, both of which inhibit the fusion between autophagosomes and lysosomes [4,133], can also be used to determine if autophagy is truly induced by the treatment of interest, since if this is the case then the level of LC3-II should increase further [17,139]. An alternative autophagy pathway not involving *ATG5*, *ATG7*, or the conversion of LC3-I to LC3-II may also occur. This pathway involves the formation of autophagosomes from the fusion of the isolation membrane with late endosomal and *trans*-Golgi vesicles regulated by the GTPase Ras-related protein 9 (Rab9) [4,140,141].

Studies Where Nanosilver Induces Autophagy:

PVP-coated 26.5 nm nanosilver was used to treat HeLa cells that had been stably transfected with LC3 tagged with enhanced green fluorescent protein (HeLa EGFP-LC3) [16]. Control HeLa EGFP-LC3 cells showed a green smear which formed into distinct bright green dots upon non-toxic nanosilver treatment, indicating LC3 aggregation. As well, the green dots for EGFP-LC3 colocalized with acidic vesicles and lysosomes as seen with the monodansylcadaverine and LysoTracker Red dyes respectively. The protein expression of LC3-II increased, TEM imaging indicated the increased presence of autophagosomes, p62 protein expression decreased, and the use of autolysosomal degradation inhibitors resulted in an increase in LC3-II in nanosilver treated HeLa cells. Together, all these factors indicated that non-toxic nanosilver exposure induced an

increase in autophagy. Inhibition of class III phosphatidylinositol 3-kinase (PtdIns3K), a kinase involved in autophagosome formation, by wortmannin inhibited the nanosilver induced increase in LC3-II expression; thus indicating that nanosilver induces autophagy through the PtdIns3K pathway. Nanosilver treatment did not change the phosphorylation level of mTOR or of its substrate ribosomal protein S6 kinase 70kDa (P70S6K) indicating that this pathway was not involved. However, Zhu *et al.* [17] did observe a decrease in phosphorylated mTOR with nanosilver treatment. *ATG5* is known to be essential for autophagy, and inhibition of autophagy by wortmannin or *ATG5* knockdown increased the nanosilver induced cell death in HeLa cells indicating that the process of autophagy was aiding in cell survival [16]. In order to understand a possible mechanism behind nanosilver induced cytoprotective autophagy, Lin *et al.* [44] examined the effects of nanosilver treatment on TFEB. HeLa cells stably transfected with TFEB tagged to enhanced green fluorescent protein (EGFP-TFEB) were treated with 26.8 nm PVP-coated nanosilver at non-toxic levels, and TFEB was seen to locate in the nucleus. The mRNA expression of the autophagy related genes MAP1LC3B and SQDTM1 were also up-regulated. Treatment with the mTOR inhibitor, Torin 1, also resulted in the translocation of TFEB to the nucleus. However, nanosilver did not appear to inactive mTORC1, ERK1/2, or PKC in this study. A time course experiment indicated that nanosilver induced TFEB translocation preceded an increase in LC3-II protein expression, and knockdown of TFEB decreased the increase in LC3-II. As well, inhibition of autophagy by 3-MA did not affect the nanosilver induced translocation of TFEB to the nucleus. Nanosilver induced apoptosis increased in the HeLa cells with both autophagy inhibition by 3-MA and TFEB knockdown, indicating the mechanism by which nanosilver induced autophagy was cytoprotective. Co-treatment of the cells with both nanosilver and the antioxidant NAC attenuated TFEB translocation, demonstrating the importance of ROS in the nanosilver induced autophagy in

HeLa cells [44]. Similarly, in human U251 glioblastoma cells, treatment with PVP-coated nanosilver (2.43, 15.47, 40.05 nm) induced autophagy in the cells [43]. This was indicated by an increase in autophagosomes identified in TEM images, an increase in florescent dots when the cells were stained with an autophagosome dye (Cyto-ID Green), the co-localization of many of the Cyto-ID fluorescent dots with that of Lyso Tracker Red indicating the presence of autolysosomes, an increase in LC3-II protein expression, and the degradation of p62. The induction of autophagy in response to nanosilver treatment was a protective mechanism for the cells, since co-treatments of nanosilver with 3-methylamphetamine (3-MA) inhibited autophagy by inhibiting PtdIns3K and resulted in increased cell death. Additionally, the ERK and JNK pathways were found to be involved in the process of nanosilver induced autophagy since inhibitors for JNK and ERK inhibited autophagy [43]. Autophagy was increased in U251 glioblastoma cells with non-toxic nanosilver (citrate-coated, 15.26 nm) treatment as seen with an increase in AVOs and LC3. Nanosilver and radiation treatment combined further increased autophagy in the cells as a survival mechanism in response to the treatments [64]. An increase in the number of AVOs were also observed in MCF-7 cells treated with non-toxic levels of 10-30 nm nanosilver [84]. Further studies with U251 glioblastoma cells by Wu *et al.* [43] indicated that non-toxic nanosilver (PVP-coated, 15.38 nm) treatment produced an increase in ROS as measured with H_2DCFDA, and this increase in ROS triggered autophagy, decreased mitochondrial membrane potential, and increased apoptosis due to radiation exposure. Antioxidant treatment with NAC or vitamin C attenuated each of these effects: decreasing the amount of ROS, LC3-II protein expression, the number of autophagosomes, and decreasing the apoptosis caused by nanosilver and radiation treatment. The inhibition of autophagy with 3-MA decreased this cell survival mechanism and lead to increased

ROS and caspase-3 activity, which increased the cell death of the glioma cells due to nanosilver and radiation [43].

Ba/F3 treated with toxic levels of PVP-coated, 11.17 nm nanosilver just above the EC_{50} level, induced autophagy in a ROS dependent manor [17]. An increased number of autophagosomes were seen with TEM, and the protein expression of LC3-II increased. Interestingly, the increase in LC3-II appeared to be nanosilver specific since this effect was not induced by silver ions. Inhibition of autolysosome degradation with chloroquine further increased the level of LC3-II, and the protein expression of p62 decreased with nanosilver treatment, indicating that autophagy was truly induced. As an additional check for normal autophagic flux, the activity of a lysosome enzyme, acid phosphatase, was examined and found to be not effected by nanosilver treatment indicating that normal lysosomal degradation occurred. ROS increased with nanosilver treatment as seen with the H_2DCF-DA dye, and pre-treatment with the antioxidants NAC or vitamin C decreased autophagy indicating that oxidative stress was upstream of this process. Nanosilver treatment greatly decreased the protein expression of active phosphorylated mTOR, and antioxidant pre-treatment relieved this inhibition. Inhibition of autophagy with *ATG5* knockdown or 3-MA co-treatment reduced the apoptosis due to the nanosilver treatment around the EC_{50} concentration. Thus, in this study, the toxic nanosilver treatment induced ROS, which inactivated mTOR, leading to the induction of autophagy, and cell death in the Ba/F3 cells [17].

Nanosilver aggregates were observed inside autophagosomes, and the protein expression of LC3-II increased in HGFs treated with 30 nm nanosilver that had been prepare in a solution of Chitlac [88]. Autophagosome accumulation and increased LC3-II protein expression was seen in HepG2

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cells treated with non-toxic nanosilver treatment (PVP-coated, 10, 50, and 100 nm). Additionally, pre-treatment with the autophagosome inhibitor, 3-MA, decreased the expression of LC3-II. Increased nanosilver treatment concentrations resulted in increased lysosome activity and dysfunction, and toxic nanosilver treatment resulted in an increase in caspase-3 activity and apoptosis. As expected, the strength of the effect elicited by the nanosilver decreased as the size of the particle increased [61].

The expression of several the *ATG* genes has been found to change in cells treated with nanosilver. In a study by Zhang *et al.* [66], TM3 and TM4 cells treated with nanosilver (10 and 20 nm) at the EC_{50} had an increased number of autophagosomes as visualized with TEM. In the TM3 cells the mRNA expression for *ATG7* and *ATG8* were up-regulated while *ATG6* was down-regulated; and in TM4 cells *ATG6* and *ATG7* were up-regulated while *ATG8* was down-regulated [66]. Nanosilver (18 nm) treatment of A2780 at the EC25 level induced the formation of many vacuoles and autophagosomes seen with TEM, as well as the up-regulating the mRNA expression of *ATG5* and *ATG7*, and down-regulating the mRNA expression of *ATG3*, *ATG6*, and *ATG10* [71]. An increase in LC3 fluorescence and the up-regulation of the autophagy genes *ATG12* and Beclin1 was observed in C17.2 cells treated with non-toxic nanosilver treatment using 4.2 nm MUA or dodecylamine-modified poly(isobutylene-*alt*-maleic anhydride (PMA) coated nanosilver [15].

In an *in vivo* experiment, male Sprague Dawley rats exposed to nanosilver (PVP-coated, 22.32 nm) via one time interperitoneal injection of 500 mg/kg had visible phagophores, autophagosomes, and autolysosomes in their liver tissue. The expression of LC3-II as well as the presence of LC3-II protein aggregates increased in the liver tissue the day after exposure and then decreased again

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over time [65]. In another study, the levels of LC3-I and LC3-II both increased with increasing nanosilver dose, but the ratio of LC3-II to LC3-I only increased at the highest treatment of 200 mg/kg/day in male Sprague Dawley rats exposed to PVP-coated nanosilver (20-30 nm) over 90 days by gavage administration [69].

Studies where Nanosilver Blocks Autophagic Flux:

On the other hand, nanosilver treatment resulted in a blockage in autophagic flux under the experimental conditions used in the following studies. NIH 3T3 cells treated with 26.2 nm nanosilver had an increase in the number of autophagosomes as seen with TEM microscopy, an increase in cytosolic AVOs, and an increase in LC3-II protein expression. However, the protein expression of p62 also increased indicating that the nanosilver treatment inhibited autophagosome turnover [63]. THP-1 monocytes also showed a blockage in autophagic flux with nanosilver treatment [138]. Autophagy is essential for the process of monocyte differentiation into macrophages, and differentiation stimulated by phorbol 12-myristate 13-acetate (PMA) was inhibited by the nanosilver (>30 nm) treatment. The protein expression of p62 increased along with the protein expression of LC3-II and the number of autophagosomes with PMA and nanosilver treatment, and degradation of p62 did not occur. Additionally, lysosome membrane stability decreased and lysosomal pH alkalization increased with nanosilver treatment, indicating lysosomal dysfunction in the THP-1 monocytes which may have played a part in the observed blockage in autophagic flux [138]. A549 cells treated with toxic levels of nanosilver (10 nm) at the EC_{50} showed an increase in the number of autophagosomes and autolysosomes, increased mitochondrial damage, and increased ATG5, LC3-II, and p62 protein expression levels; which indicated a blockage in autophagic flux in these cells. Interestingly, pre-exposure of the A549 cells

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to hypoxia before the EC_{50} nanosilver treatment reduced the nanosilver induced increase in p62, while still increasing ATG5 and LC3-II. HIF-1α knockdown decreased the nanosilver induced increase in the levels of ATG5, LC3-II, and p62, indicating the importance of HIF-1a in mediating autophagy [85]. In another study, non-toxic nanosilver (citrate-coated, 69.8 nm) treatment of A549 cells increased the number of autophagic vacuoles seen with Cyto-ID fluorescence and increased the protein expression of LC3-II [133]. This increase was due to a blockage in autophagic flux since the level of p62 and also increased, and inhibition of autophagy by bafilomycin A1 did not cause a further increase in LC3-II. Contrary to the study by Lin *et al*. [44], nanosilver treatment decreased the protein and mRNA expression of TFEB, and overexpression or knockdown of TFEB in nanosilver treated cells did not change the level of LC3-II. Lysosomal pH was found to increase indicating that lysosomal dysfunction occurred in this study [133].

Nanosilver induced blockage of autophagic flux was also seen in human pulmonary mucoepidermoid carcinoma (NCI-H292) cells treated with citrate-coated or lipoic acid coated nanosilver (10 nm), where an increased number of autophagosomes were seen as well as increased protein expression of LC3-II and p62 [4]. However, it was found that nanosilver treatment of transformed human bronchial epithelial (BEAS-2B) cells increased the number of autophagosomes containing Rab9, indicating that alternative autophagy was induced even though conventional autophagy was blocked by the nanosilver treatment [4].

Nanosilver and Angiogenesis

Angiogenesis is the natural process of new blood vessels growing from the capillary endothelium of established blood vessels [142,143]. This is especially important during physiological growth

and development, or during wound healing [144]. Unfortunately, cancer takes advantage of this system, stimulating increased angiogenesis in order to bring in more oxygen and nutrients to the tumor and enabling it to grow and spread [142,144]. Angiogenesis is also involved in several ischemic, ocular, and inflammatory diseases [144,145]. An imbalance in the expression of pro-angiogenic growth factors such as VEGF, acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2), and angiopoietin; versus angiogenesis inhibitors such as endostatin and angiostatin, leads to disease. The process of angiogenesis involves the production of growth factors, VEGF and FGF-2 stimulated release of proteases and plasminogen activators from the endothelial cells to degrade the basement membrane of the blood vessel, cell migration into the surrounding tissue, and cell proliferation to form the new blood vessels. One of the pathways mediated by VEGF in angiogenesis is the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway. VEGF binds to and activates the VEGF receptor (VEGFR, also known as KDR or Flk-1), a type III receptor tyrosine kinase, which in turn activates phosphatidylinositide 3-kinase (PI3K), which leads to the phosphorylation and activation of AKT, a serine/threonine kinase involved in cell survival and the mTOR pathway [144,146]. Other proteins phosphorylated by VEGFR include focal adhesion kinase (FAK), ER retention defective 1 (ERD1), ER retention defective 2 (ERD2), p38 mitogen-activated protein kinase, and endothelial nitric-oxide synthase (eNOS) [147].

Gurunathan *et al.* [144] found that treatments of bovine retinal endothelial cells (BREC) with concentrations of 40-50 nm nanosilver up to the EC_{50} value for the cells decreased angiogenesis via inhibition of the PI3K/AKT pathway. VEGF treatment was used to induce angiogenesis and compared to co-treatments of nanosilver with VEGF, which greatly decreased the VEGF

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induction. Nanosilver treatment dramatically decreased VEGF induced cell proliferation, cell migration, capillary-like tube formation, PI3K activity, and AKT phosphorylation [144]. In a following study, nanosilver EC_{50} treatment of the BREC cells induced apoptosis through caspase-3 activation [148]. In a study using HUVEC cells, toxic nanosilver treatment with an average size of 10 nm resulted in greatly decreased the protein level of VEGF in the cells, as well as endothelial tube formation [57].

Sheikpranbabu *et al.* [149] examined the effects of non-toxic 50 nm nanosilver treatments on endothelial cell permeability using porcine retinal endothelial cells (PREC). VEGF stimulates endothelial cell permeability, as does the inflammatory cytokine IL-1β. Src kinase is also involved in angiogenesis, and higher levels of active phosphorylated Src kinase are found with VEGF induced vascular permeability [150]. Non-toxic nanosilver treatment significantly inhibited VEGF and IL-1β from increasing the levels of phosphorylated Src kinase, and in this way decreased the VEGF and IL-1β induced permeability of the PREC cells [149]. In terms of endothelial cell viability and proliferation with nanosilver treatment, Castiglioni *et al.* [151] reported that non-toxic 35 nm nanosilver treatment of human microvascular endothelial cells (HMEC) decreased the cellular proliferation while not effecting cellular viability. This inhibition did not permanently affect the cells since cell proliferation increased again after the removal of the nanosilver [151]. Female Wistar rats treated with intraperitoneal injection of 50-60 nm nanosilver resulted in decreased angiogenesis in the ovarian tissue visualized via immunofluorescence [152]. In a wound healing study using NHDF cells and NHEK cells, decreased production of VEGF was seen for both low and high treatment concentrations of 10 nm nanosilver, indicating decreased angiogenesis, with a greater inhibition of VEGF production seen in NHEK cells [91]. Wounds on

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male Wistar rats healed soonest with treatment of non-toxic doses of 10-20 nm nanosilver, followed by rats treated with toxic doses of nanosilver, the positive control rats treated with silver alginate cream, and finally the negative control rats with no treatment healing last. Histological analysis of the wounds revealed decreased angiogenesis in the wounds for the rats treated with the non-toxic nanosilver dose. Thus non-toxic nanosilver treatment of wounds was found to aid in healing [99,100].

Anti-angiogenesis effects of nanosilver have also been reported using *in vivo* angiogenesis models such as the chick chorioallantoic membrane assay (CAM), Matrigel implant, and aortic ring models. Decreased angiogenesis in the mouse matrigel model and the CAM model was observed with 10-30 nm nanosilver capped with diaminopyridinyl (DAP)-derivatized heparin (HP) polysaccharide (DAPHP) when angiogenesis was induced using FGF-2. Male mice (C57BL/6NCr) subcutaneously injected with Matrigel containing FGF-2 and DAPHP-capped nanosilver for 12 days had decreased hemoglobin content in the Matrigel with the nanosilver as compared to the Matrigel with just FGF-2, indicating decreased angiogenesis. Additionally in the CAM model, the number of FGF-2 induced new blood vessel branch points was seen to decrease with co-treatment of DAPHP-capped nanosilver [153]. Treatment with 16.5 nm nanosilver resulted in decreased blood vessel formation, decreased hemoglobin content, and smaller chick embryos in the CAM assay [154]; and 12 nm nanosilver treatment resulted in smaller and fewer blood vessels formed in a rat aortic ring model [155]. Interestingly, treatment with larger 100 nm nanosilver did not significantly inhibit angiogenesis in the CAM assay showing the importance that the size has on the effect [156].

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The only report of nanosilver increasing angiogenesis is by Kang *et al.* [147], who found increased blood vessel formation and hemoglobin content with the Matrigel assay in female C57BL/6 mice. In this study, angiogenesis was not induced with growth factor treatment, and the Matrigel was only mixed with the 2.3 nm PVP–coated nanosilver. After 10 days the Matrigel implants had increased hemoglobin content compared to the controls. SVEC4-10 mouse endothelial cells had increased levels of VEGF and nitric oxide with the nanosilver treatment; as well as increased levels of phosphorylated and active FAK, AKT, ERK1/2, and p38, indicating increased VEGFR signalling [147]. Some of the key differences between this study reporting increased angiogenesis and all the other studies reporting decreased angiogenesis are: angiogenesis was not induced by VEGF or FGF-2 along with the nanosilver treatment, a smaller size of nanosilver particles was used, differences in the strain and sex of mice or type of cells used, or different treatment doses and times. No cell viability assay data is included in this study, so the toxicity of the treatments cannot be determined or commented on. However, the treatment concentrations used were in the same range that has been used in other studies with different cell types. Thus, with the exception of the work by Kang *et al.* [147], the consensus of the studies to date is that nanosilver treatment inhibits angiogenesis.

Nanosilver and Epigenetics

Epigenetics refers to the heritable and reversible modifications that customize gene expression without involving mutation of the DNA sequence. These consist of DNA methylation, histone tail modifications, and post-transcriptional regulation by non-coding RNAs [157,158].

Nanosilver and DNA Methylation:

DNA methylation in the promotor region of a gene generally represses transcription, while demethylation of the promotor region allows for transcription of the gene. Methylation may occur on adenine at the N-6 position in a 5'-G-A-T-C-3' sequence or on cytosine at the C-5 position in a 5'-C-G-3' (CpG) sequence. The methyl group is transferred from S-adenosylmethione (SAM) to the cytosine residue by DNA methyltransferases (DNMTs) [159]. DNA methylation on cytosine forms 5-methylcytosine (5-mc). This methylation inhibits the transcriptional machinery from accessing the DNA, as well as attracting methyl-CpG-binding domain (MBD) proteins which bind and further block transcription. DNA demethylation is not well understood, however, the transformation of 5-mc to 5-hydroxymethylcytosine (5-hmc) by the 5-methylcytosine hydroxylase TET1 (ten-eleven translocation 1) enzyme produces another important form of cytosine that plays a part in the demethylation process [157,158]. Methylation of the cytosine residues at CpG sites within the gene are also involved in gene regulation. Highly expressed genes have low promotor methylation and high gene-body methylation, while low expressed genes are methylated more in the promotor region and less in the body of the gene [160].

Blanco *et al.* [161] examined the effects of nanosilver on global DNA methylation using A549 cells, and found an increase in methylation after treatment with 21.74 nm PVP-coated nanosilver, however, the treatment used in this study was toxic to the cells. Increased amounts of 5-mC and of the DNMT enzymes were found in mouse hippocampal neuronal (HT22) cells after 48hr nanosilver (~8 nm) treatment at the EC_{50}. Interestingly, elevated levels of 5-mC and DNMTs (especially DNMT2) were still seen even after the toxic nanosilver treatment was removed and the cells allowed to recover in media for an additional 96hrs [162]. DNA methylation changes were also found on several genes extracted from the placentas of pregnant mice that had been treated

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intravenously with 8nm nanosilver. Specifically, methylation of the pleiomorphic adenoma gene-like 1 (*Zac1*) gene decreased, while methylation of the insulin-like growth factor 2 receptor (*Igf2r*) gene was marginally increased [7].

Nanosilver and Histone Tail Modifications:

DNA is wrapped around histone proteins forming nucleosomes. Eight histone proteins form each nucleosome core, two each of H3, H4, H2A, and H2B, and these wrap approximately 147 base pairs of DNA [163]. The N-terminal tails of the histone proteins extend beyond the nucleosome core and are subject to various covalent modifications which determine how tightly the DNA is packed. These modifications include acetylation, phosphorylation, methylation, ubiquitination, and SUMOylation. Histone tail lysine acetylation by histone acetyltransferases (HATs) open the DNA allowing increased expression, while deacetylation by histone deacetylases (HDACs) condenses the DNA making it inaccessible for expression [158]. Methylation of lysine or arginine residues in the histone tails either relax or condense the DNA depending on the combination of residues that are methylated. For example, trimethylation of lysine 4 on H3 (H3K4me3) and monomethylation of lysine 79 on H3 (H3K79me1) allows for active gene transcription, and phosphorylation of serine 10 on H3 (p-H3S10) corresponds to condensation of the DNA [164]. Additionally, phosphorylation of serine 139 on histone H2A.X (γ-H2A.X) is an indication of DNA damage due to double-strand breaks [165]. Histone tail modifications and DNA methylation are also important in determining where the nucleosome is formed on the DNA, and thus greatly affect gene expression [19,157].

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Currently, studies examining the effects of nanosilver on histone tail modifications in cells have only looked at methylation, phosphorylation and acetylation. Non-toxic nanosilver treatment of MEL cells with 25nm PVP-coated nanosilver (1, 8 μg/mL) decreased the global methylation on histone 3 [19]. These erythroid precursor cells were differentiated into red blood cells by DMSO treatment, and the β-globin locus specifically examined. Western Blot indicated that the overall levels of H3K4me3 and H3K79me1 were decreased in cells treated with nanosilver, while ChIP-PCR revealed that H3K4me3 and H3K79me1 decreased by at least 50% at the β-globin locus. The levels of histone methyltransferase enzymes: myeloid/lymphoid or mixed-lineage leukemia 2 (MLL2) involved in H3K4 trimethylation, and disruptor of telomere silencing 1 (Dot-1L) for H3K79 methylation, were also reduced. As well, pull-down and immunoprecipitation assays indicated that the nanosilver bound directly to the H3 and H4 histones. Not surprisingly, all this resulted in reduced RNA polymerase II activity at this locus, lower levels of β-globin mRNA and protein, and reduced amounts of hemoglobin produced in the cells [19].

Nanosilver treatment (≥100 nm, ~1 mg/mL) of MCF-7 cells, A549 cells, and human skin keratinocytes (HaCaT) increased the serine 10 phosphorylation H3 [164,166]. This occurred though nanosilver induced activation of Aurora kinases (AURKs) which regulate H3S10 phosphorylation. Although more study is needed, Zhao *et al.* [164] suggests that the uptake of nanosilver allows for the release of silver ions into the cell, which then trigger the release of globular actin (G-actin) from filament actin (F-actin). This rearrangement of the cytoskeleton activates AURKs which phosphorylate H3S10 as part of the cellular response [164]. Histone H2A.x phosphorylation has been reported with both toxic and non-toxic nanosilver treatment

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concentrations in MCF-7 and A549 cells [74,161], and deacetylation of H3 occurred with toxic nanosilver treatment in A549 cells with PVP-coated nanosilver (21.74 nm) [161].

Nanosilver and Non-coding RNA Regulation:

Post-transcriptional regulation by non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are also involved in epigenetics [157,158]. MicroRNAs are short (22-25 nucleotides) single stranded non-coding RNAs that bind to target mRNA and either cause them to be degraded if there is complete complementarity between the miRNA and the mRNA, or cause the expression to be inhibited if there is incomplete complementarity [158]. Long non-coding RNAs are non-coding RNAs longer than 200 nucleotides. They serve to regulate miRNA action, mRNA gene expression, protein function, and cellular functioning [73].

Several studies have been done examining the effect of nanosilver exposure on post-transcriptional regulation by non-coding RNAs, with one study examining the effect on lncRNA regulation, and several studies examining the effect on miRNA regulation. The effect of nanosilver exposure on lncRNA regulation was examined using K562 cells which were treated with 11, 27, and 95 nm PVP-coated nanosilver [73]. Non-toxic nanosilver treatment significantly up- or down-regulated several lncRNAs, with osteosarcoma doxorubicin-resistance related up-regulated lncRNA (ODRUL) being up-regulated the most by approximately tenfold. A miRNA microarray with human Jurkat T cells clone E6-1 revealed that the expression of 19 miRNAs were up-regulated and 44 miRNAs were down-regulated by 24hr treatment with 0.2μg/mL ≥100 nm nanosilver. Messenger RNA microarray data was compared and decreased expression of hsa-miR-219-5p miRNA corresponded with an increase in MT1F and tribbles homolog 3 (TRIB3) mRNA. Network

analysis of the interactions between the miR-219-5p miRNA and the MT1F and TRIB3 mRNA indicated that pathways involved in oxidative stress response, cell cycle, cell survival, and apoptosis would be effected [167]. Human embryonic stem cell-derived neural stem/progenitor cells (hESC-derived NPCs) are commonly used to study developmental neurotoxicity. In a study by Oh *et al.* [3], hESC-derived NPCs were treated with 13.3 nm citrate-coated nanosilver and miRNA and mRNA microarrays were evaluated. At 6 hrs, the nanosilver treatment of 25 µg/mL was non-toxic to the cells, and 11 miRNAs were up-regulated while 20 mi-RNAs were down-regulated, affecting the mRNA expression of 611 genes. At 24 hrs, the treatment was slightly toxic to the cells and 22 miRNAs were up-regulated while 10 miRNAs were down-regulated, affecting the mRNA expression of 280 genes. The regulation of only 6 miRNAs and the mRNA expression of only 33 genes were the same for both treatment times. Analysis of the target genes affected by the changes in miRNA regulation due to the nanosilver treatment indicated that pathways involving Nrf2 mediated oxidative stress response were strongly affected at both treatment times. Neuronal function, neuronal signalling, and nervous system development were affected by the 6 hr treatment, and the inflammation response pathway was affected by the 24 hr treatment [3]. Treatment of human dermal fibroblasts (HDF) with non-toxic levels of 20 nm nanosilver (200 µM) changed the regulation of 246 miRNAs and the expression of 25 proteins, affecting numerous cellular pathways, and leading to actin cytoskeleton damage, a decrease in intracellular ATP, and activation of apoptosis [139].

In a different model system, the exposure of nematodes to 58.3 nm PVP-coated nanosilver treatment at the EC_{30} for ten generations resulted in an approximately 10-fold sensitization that did not dissipate in the subsequent generations that were also exposed to nanosilver. Nematodes

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exposed to nanosilver for only five generations retained the sensitivity in later generations even though they were no longer exposed to the nanosilver, indicating epigenetic effects [24]. Thus, epigenetic changes in terms of increased DNA methylation, various histone tail modifications, and changes in non-coding RNA expression were seen with nanosilver treatment.

Nanosilver and Genotoxicity

Genotoxicity refers to damage to a cell's DNA or RNA by a genotoxic agent [168,169]. This DNA damage could include mutations, DNA adduct formation, oxidation of the DNA bases, single or double strand breaks, the formation of crosslinks, and structural changes. Genotoxic nanoparticles can induce DNA damage either directly through DNA binding; or indirectly through binding to associated proteins, the mitotic spindle components, or producing oxidative stress [170]. The main endpoints that need to be evaluated in order to determine genotoxicity are the presence of DNA damage, structural and numerical chromosomal aberrations, and mutations. Some DNA and RNA damage may be able to be repaired by the cellular repair mechanisms. However, chromosomal aberrations and mutations are irreversible and may lead to cancer, or to heritable diseases if the damage occurs in the germ cells [168,169,171]. Many *in vitro* genotoxicity assays may be done, however, these *in vitro* tests tend to be highly sensitive but not very specific and give many false positive results making follow up *in vivo* testing necessary for confirmation [169]. Common *in vitro* genotoxicity tests include the Ames test for mutagenicity in bacteria, the alkaline single-cell microgel electrophoresis (comet) assay for DNA damage, the *in vitro* mammalian chromosomal aberration test, the *in vitro* mammalian cell micronucleus test, and the *in vitro* mammalian cell gene mutation test (thymidine kinase (TK) mouse lymphoma assay (MLA), or hypoxanthine phosphoribosyl transferase (HPRT) gene mutation assays) [169,172]. The comet assay detects

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DNA single and double strand breaks, incomplete excision repair sites, alkali labile sites, and DNA base oxidation [54,84,170]. Histone H2A.X phosphorylation on serine 139 is an early indication of DNA double strand breaks [165]. DNA strand breaks can also be detecting using Hoechst staining [87]. The presence of DNA adducts can be detected with 32P-postlabeling, and the levels of 8-oxoguanine may be used as an indication of the levels of DNA base oxidation since 8-oxoguanine is the main purine oxidation product [170]. If not repaired, 8-oxoguanine may lead to the formation of transversion mutations [48]. The majority of studies both *in vitro* and *in vivo* indicate that nanosilver has genotoxic effects; however, other studies do not report any genotoxicity.

Nanosilver and Genotoxicity In Vitro Studies:

Non-toxic nanosilver treatment of hMSC cells with 46 nm nanosilver resulted in the presence of nanosilver in the nucleus and induced DNA damage. The comet assay as well as the chromosome aberration test indicated genotoxicity, and the chromosome aberrations were found to consist of both chromatid deletions and chromatid exchanges [54].

DNA damage was detected with the comet assay in MCF-7 cells, and the comet tail length was significantly increased in cells treated with non-toxic concentrations of 10-30 nm nanosilver above the EC_{50} [84]. Interestingly, the genotoxicity observed by the comet assay as a result of HMEC cells treated with 35 nm nanosilver was not permanent, and was not seen once the treatment was stopped and the cells were allowed time to recover [151]. Nanosilver (BSA-coated, 15.9 nm) treatment induced an increase in the number of DNA adducts in CHO-K1 cells at low treatment concentrations, while higher treatment concentrations were needed in order to observe an increase

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in 8-oxoguanine levels and micronuclei formation [62]. An increase in 8-oxoguanine in human Chang liver cells treated with nanosilver (5-10 nm) treatment was accompanied by a decrease in both the gene and protein expression of 8-oxoguanine DNA glycosylase 1 (OGG1), a base excision repair enzyme that excises and repairs 8-oxoguanine [173]. DNA damage via the comet assay and micronuclei formation were observed in U251 and IMR-90 cells treated with starch-coated 6-20 nm nanosilver [60]; as well as in Chinese hamster ovary cells treated with ≥100 nm nanosilver [174]. HepG2 cells treated with non-toxic concentrations of nanosilver (7-10 nm, polyethylenimine-coated) resulted in micronuclei formation in the cells. In order to try to separate the effects due to the nanosilver particle itself and the Ag^+ ions, cysteine was added to bind the Ag^+ ions. This inhibited cell death and reduced but did not eliminate the micronuclei formation in the cells, indicating that both the nanosilver and the Ag^+ ions affected that endpoint. DNA microarray data indicated the up-regulation of several stress responsive genes, such as for MT and heat shock proteins, as well as for genes involved in DNA repair such as the RAD 51 family member, RAD51C [50]. Similar results are reported by Sahu *et al.* [55] in a DNA microarray study with HepG2 cells treated with non-toxic concentrations of 20 and 50 nm nanosilver, with genes for MTs, heat shock proteins, and DNA repair (such as growth arrest and DNA damage-inducible 45 (GADD45)) being upregulated [55]. The protein expression of RAD51 also increased in TM3 cells and TM4 cells treated with nanosilver (10 and 20 nm) at the EC_{50} level [66,175]. An increase in γ-H2A.X histone phosphorylation at serine 139 was found with non-toxic nanosilver treatment (≥100nm, 1 mg/mL) in MCF-7 cells and was thought to be the result of double strand breaks from the nanosilver treatment due to the time needed before the phosphorylation occurred [74]. Similarly, an increased level of γ-H2A.X phosphorylation was seen in A549 cells with both toxic

and non-toxic treatments PVP-coated nanosilver (21.74 nm) [161]. DNA damage due to double strand breaks was also indicated in HepG2 and Caco-2 cells using Hoechst 33258 staining [87].

However, no increase in genotoxicity has been observed in human skin keratinocyte (HaCaT) cells treated with non-toxic levels of 30 nm citrate-coated nanosilver and tested with the comet assay [176]. As well, mutagenicity was not seen in an assay involving mouse embryonic fibroblasts (MEF-*LacZ*) containing the bacterial lacZ gene and treated with 20, 80, and 110 nm nanosilver [177]. The Ames test has been found to generally result in negative results when testing various nanoparticles [178], and was also found to be negative for mutagenicity in *Salmonella typhimurium* bacterial strains treated with nanosilver (≥100 nm) [174].

Nanosilver and Genotoxicity In Vivo Studies:

Several *in vivo* studies do not report genotoxicity; however, other *in vivo* studies using different experimental conditions or measuring alternate end points do indicate genotoxicity. In a study by Kim *et. al.* [34], male and female Sprague-Dawley rats were treated orally with nanosilver (60 nm) for 28 days at no, low (30 mg/kg), medium (300 mg/kg), and high (1000 mg/kg) treatment levels. The quantity of micronucleated polychromatic erythrocytes and the ratio of polychromatic erythrocytes to total erythrocytes did not change in the nanosilver treated rats; indicating no evidence of DNA damage and no genotoxicity to the bone marrow cells respectively. However, there was some evidence of slight liver damage in the rats treated with the highest nanosilver treatment level, as the alkaline phosphatase and cholesterol levels were affected in these rats [34]. In another *in vivo* study by Kim *et. al.* [179], using a longer treatment time and inhalation instead of oral exposure, male and female Sprague-Dawley rats were exposed to nanosilver (18 nm) by

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inhalation for 90 days at low (0.7 x 106 particles/cm^3), medium (1.4 x 106 particles/cm^3), and high (2.9 x 106 particles/cm^3) exposure amounts. Once again, there was no change observed in the frequency of micronucleated polychromatic erythrocytes, and the ratio of polychromatic erythrocytes to total erythrocytes did not change, indicating no genotoxic affects in the rat bone marrow [179]. Wen *et al.* also found no increase in bone marrow micronuclei in female Sprague-Dawley rats treated with nanosilver (6.3-629 nm, 5 mg/kg) intravenously for 24 hr. However, there was a 14.3% increase in aberration cells, a 7.1% increase in multiple aberration cells, and a 4.3% increase in the number of polyploidy cells in the nanosilver treated rats. Treatment with Ag^+ ions (0.0003 mg/kg) resulted in a higher increase in aberration cells and multiple aberration cells, but only 0.1% polyploidy cells, indicating either that this might be a nanosilver specific effect or that the low amount of polyploidy cells in the Ag^+ ion treated rats may be due to chromosome fragmentation [180]. Patlolla *et al.* [181] did observed increased micronuclei formation, as well as structural chromosome aberrations, DNA damage as seen via the comet assay, and a decrease in cell division measured by the mitotic index in bone marrow cells of male Sprague-Dawley rats treated orally with 10 nm nanosilver (5, 25, 50, 100 mg/kg) once a day for 5 days. The higher doses (50 and 100 mg/kg) induced a significant increase in all of the endpoints tested, as well as resulting in an increase in ROS as measured by H_2DCFDA [181]. Examining the genotoxicity of nanosilver with different coatings, Nallanthighal *et al.* [48] orally treated male and female C57BL/6J *pun/pun* mice with 20 nm citrate or PVP coated nanosilver (4 mg/kg) for 7 days. This dose is 800× the daily oral exposure amount in humans that is thought to be safe by the United States Environmental Protection Agency (EPA), and is in the range of Ag exposure that could cause argyria. The citrate-coated nanosilver induced DNA damage *in vivo* whereas the PVP-coated nanosilver and the Ag^+ ion control did not. Chromosomal aberrations in the form of micronuclei were detected in the bone

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marrow. As well, increased 8-oxoguanine indicating DNA oxidation, and phosphorylation of histone H2A.X indicating the presence of double strand breaks were detected in the peripheral blood leukocytes of mice treated with citrate-coated nanosilver [48]. Not surprisingly, the treatment of mice deficient in OGG1 results in increased genotoxicity in the mice [182].

Nanosilver and Cancer

Cancer in its various forms is a devastating disease, and results from a combination of environmental, physiological, and genetic factors [14]. Cancer cells are resistant to pro-death and anti-proliferative signals, are self-sufficient in terms of growth signals, and can replicate limitlessly. In order to form and grow, it is thought that cancer must first be initiated in a cell through impaired mitochondrial function or through various genetic mutations and rearrangements, resulting in irreversible changes to the cell, activated oncogenes, and dysfunctional tumor suppressor proteins [90,183,184]. Growth of the cancer cell must be promoted by the surrounding cellular environment, and if not checked by the immune system, the disease can then progress with increased tumor growth and metastasis [90]. Many current treatments have only limited effectiveness, and also produce undesired and damaging side effects. Thus, new treatments, combinations of treatments, and the incorporation of nanoparticles such as nanosilver into treatments are currently being examined [14,185]. The p53 tumor suppressor transcription factor is involved in inducing cell cycle arrest, apoptosis and senescence [71]. Activated p53 induces cell cycle arrest, and one of the downstream genes activated by p53 is p21, a cyclin kinase inhibitor (CKI) involved in cell cycle arrest at the G1 phase [186]. This arrest allows for DNA repair to occur, thus preventing the replication of damaged DNA. However, if the DNA damage

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is too great and repair is not feasible, apoptosis is triggered. If cell cycle arrest and DNA repair does not occur, cancer may develop [55,69].

The Response of Cancer vs Non-cancer Cells to Nanosilver Treatment:

Cancer cells of various cell lines have been found to be more susceptible to nanosilver treatment non-cancer cells. The relative sensitivities of non-cancer and cancer cells to nanosilver was examined using primary mouse embryonic fibroblast (P-MEF) cells and immortalized mouse embryonic fibroblast (I-MEF) cells respectively, and both cell types treated with the same nanosilver treatment (20 μg/mL for 24hr). The cancer-like I-MEF cells were much more susceptible and had a drastically reduced cell viability of 28.03% compared to the non-cancerous cells which remained 91.56% viable [16]. A549, human ovarian cancer cells (2780), human breast adenocarcinoma cells (MCF-7), and human breast adenocarcinoma cells (MDA-MB 231) all demonstrated a significant decrease in cell viability when treated with 0-50 μg/ml nanosilver (10 nm); whereas normal lung epithelial cells (L132) remained completely viable in that treatment range [85]. In human breast cells, treatment with 2 and 15 nm nanosilver produced much more cell death in cancerous MCF-7 and T-47D cells than in non-cancerous MCF-10A cells [119]. Interestingly, nanosilver (starch-capped, 6-20 nm) induced DNA damage to a much greater extent in cancerous human glioblastoma (U251) cells than in non-cancerous IMR-90 cells [60]. Nanosilver (23.44 nm, PVP-coated) was more cytotoxic to HepG2 cells than to normal hepatic L02 cells in terms of decreased mitochondrial membrane potential and cell membrane leakage. Interestingly, the nanosilver induced an increase in ROS as measured with H_2DCFDA in the cancerous HepG2 cells but not in the non-cancerous L02 cells, and triggered mitochondrial mediated apoptosis and cell death via the Fas death receptor pathway in the HepG2 cells [187].

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Mechanisms Involved in the Effect of Nanosilver on Cancer Cells In Vitro:

Numerous studies report the anti-cancer effects of nanosilver treatment on various cancer cells *in vitro* [86,122,156,188–192], with many pathways being involved. The expression of HIF-1α aids in cell survival under low oxygen conditions and is found to be high in cancer cells inside solid tumors. The high level of HIF-1 aids the cancer cells in their resistance against treatment and death. This was demonstrated in A549 cells with nanosilver treatment at the EC_{50} level, where pre-exposure to hypoxia in order to induce the expression of HIF-1α attenuated the nanosilver induced cell death [85]. Nanosilver has been found to work against this survival mechanism in cancerous MCF-7 cells, and treatment with 10 nm nanosilver was able to significantly inhibit the production of HIF-1 through decreasing activation of the hypoxic response element, even when this pathway was stimulated by hypoxia or by the hypoxic mimic cobalt chloride ($CoCl_2$). This decreased the protein levels of HIF-1α and HIF-2α present in the cells, reducing the expression of HIF-1 target genes such as VEGF-A and GLUT1, and serving as both an anti-cancer and anti-angiogenesis agent [57]. Cancerous human pancreas ductal adenocarcinoma (PANC-1) cells were approximately two times more susceptible to nanosilver induced cell death than non-cancerous immortalized human pancreas duct epithelial (hTERT-HPNE) cells when treated with either 2.6 nm or 18 nm nanosilver; with the smaller 2.6 nm nanosilver being more toxic to both cell lines than the larger 18 nm nanosilver. Early apoptosis was mainly induced in the cancerous PANC-1 cells with nanosilver concentrations up to the EC_{50} concentration, above which late apoptosis and necroptosis began increasing. In PANC-1 cells, nanosilver increased the protein expression of LCS-II potentially indicating the induction of autophagy. Necroptosis was also induced with an increase in protein expression of receptor-interacting serine/threonine-protein kinase 1 (RIP1),

receptor-interacting serine/threonine-protein kinase 3 (RIP3), and mixed lineage kinase domain-like pseudokinase (MLKL). The pro-apoptotic protein Bax increased in expression, while the anti-apoptotic protein Bcl-2 decreased in expression. As well, the protein expression of p53 also increased [51]. In HepG2 cells treated with a non-toxic concentration of nanosilver (20 nm), analysis of DNA microarray data revealed that many genes involved in the cancer pathway were up-regulated. As well, TGF-β, MAPK, and the p53 signalling pathways were all up-regulated and formed an intracellular signalling cascade [55]. In fact, p53 signalling was found to be essential for the mitochondrial mediated apoptosis via the MAPK signalling cascade that was triggered by high nanosilver (20 nm) treatment in HCT116 cells [122]. Buttacavoli *et al.* [14] conducted an integrated proteomic study exploring the biochemical mechanisms in cancer cells affected by treatment with biosynthesized 11 nm nanosilver embedded in a specific polysaccharide (extracellular polymeric substance (EPS)) via the bacteria *Klebsiella oxytoca* DSM 29614. The biosynthesized nanosilver was tested on human breast cancer cells SKBR3 and 8701-BC, and human colon cancer cells HT-29, HCT 116, and Caco-2, and found to be cytotoxic to all of the cancer cells tested, with the SKBR3 cells being the most susceptible. Some of the treated SKBR3 cells underwent apoptosis (11%), and evidence of activated autophagy was observed. The cytotoxicity of the nanosilver on the SKBR3 cells was compared to the effect on non-cancerous HB2 mammary epithelial cells, with the nanosilver treatment being selectively toxic to the cancer cells and having a selectivity index similar to the commonly used chemotherapy drug doxorubicin. Nanosilver treatment of the SKBR3 cells at both a non-toxic concentration and at the EC_{50} concentration greatly decreased the cell motility and colony forming capacity of the cancer cells. MMP enzymes are important in the process of cell migration, and the activity and protein levels of MMP-2 and MMP-9 decreased with EC_{50} nanosilver treatment. Differential gel electrophoresis

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(2D-DIGE), proteomic identification, and analysis using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database for protein-protein interactions and the DAVID bioinformatics resource for pathway identification, together indicated that the nanosilver treatment mainly affected the expression of proteins involved in pathways in the mitochondria, ER, oxidative stress response, apoptosis regulation, and down-regulated enzymes involved in glycolysis [14]. A proteomics study by Verano-Braga *et al.* [40] using human colon carcinoma (LoVo) cells found that exposure to 20 nm citrate-coated nanosilver induced protein carbonylation and ROS as measured with H_2DCFDA. The proteomics results were analyzed with Gene Ontology and STRING and were corrected for affects by released Ag^+ ions in order to examine the nanosilver particle effect only. Proteins involved in the proteasome, translation initiation, oxidative stress response, and cell death were up-regulated. Mitochondrial electron transport chain proteins decreased, either through down-regulation or degradation, as did proteins involved in cell growth, spliceosome function, and mitochondrial translation. Specifically, small ubiquitin-related modifier 2 (SUMO2), a member of the SUMO protein family, and tripartite motif containing 28 (TRIM28), an E3 ligase involved in SUMOylation, were both up-regulated by the nanosilver treatment, indicating activation of the SUMO pathway. However, the SUMOylation protein targets could not be identified in this study [40].

Cancer in vivo *Studies with Nanosilver Treatment*

In a study by Sriram *et al.* [193], Dalton's lymphoma ascites (DLA) cells were used to produce tumors in female Swiss albino mice. Then starting the following day, the mice were treated with 500 nM nanosilver (50 nm) via intraperitoneal injection for 15 days, with the treatment concentration of 500 nM being the EC_{50} for DLA cells. Not surprisingly, this high of a treatment

concentration activated caspase-3, induced DNA fragmentation, and lead to apoptosis in the DLA cells. However, in the non-cancerous control mice that were treated with 500 nM nanosilver there were no signs of toxicity such as appetite loss, reduction in body weight, fatigue, or fur colour change that were seen. The tumor-bearing mice were greatly helped by the nanosilver treatment. They had significantly fewer malignant DLA cells in their peritoneal fluid and they lived for 50% longer than the untreated tumor-bearing mice. As well, the nanosilver treatment decreased the excess ascetic fluid by 65%, bringing the body weight and white blood cell and platelet counts in the ascetic fluid back to normal. Thus in this study, nanosilver treatment successfully inhibited the cancer growth without causing adverse effects in the mice [193]. One-time nanosilver (citrate-coated, 5 nm) treatment via peritumoral injection around tumors made from murine lung squamous tumor cells (KLN 205) in female immune competent DBA/2 mice and immune deficient NOD SCIDγ mice initially reduced the size of the tumors in both types of mice. After this treatment, the effects of the nanosilver gradually dissipated in the tumors in the immune deficient NOD SCIDγ mice, and the tumor growth rate gradually increased back to the initial growth rate. The growth rate of the tumors in the immune competent DBA/2 mice was greatly reduced after the nanosilver treatment and did not recover, possibly due to the immune response triggered by the nanosilver treatment in these mice [92].

Nanosilver modified with the cell penetrating TAT peptide to improve cellular uptake has been found to be an effective and promising anti-cancer treatment against both multidrug-resistant (MDR) cancer cells and non-resistant cancer cells, using MDR melanoma B16 cells, MDR breast cancer cells MCF-7/ADR, HeLa, and MCF-7 cells [194]. Both the TAT-modified nanosilver (8 nm) and the un-modified PVP-coated nanosilver were effective in inhibiting cancer cell

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proliferation, with the TAT modified nanosilver being 4, 7, 24, and 9 times more potent than the un-modified nanosilver on the B16, MCF-7/ADR, HeLa, and MCF-7 cells respectively. Female nude mice with MDR B16 melanoma tumors simulating late stage MDR cancer exhibited inhibition of tumor growth with nanosilver and TAT-modified nanosilver peritumoral treatment, with the TAT-modified nanosilver being as effective as the doxorubicin (DOX) treatment (>85% growth inhibition) but without the debilitating adverse effects that generally makes DOX treatment unfeasible [194].

The levels of p53, p21, and cleaved caspase-3 increased in the liver tissue from male Sprague Dawley rats treated orally with up to 100 mg/kg/day PVP-coated nanosilver (20-30 nm) for 90 days, and then decreased again at higher treatment where autophagic cell death was thought to occur [69].

Nanosilver and Radiation Treatment:

Nanosilver treatment (PVP-coated, 15.47 nm) of human U251 glioblastoma cells was found to increase cell death when used in combination with radiation treatment, and this cell death further increased when additionally combined with the autophagy inhibitor, 3-MA [42]. In HepG2 cells, nanosilver treatment increased the effectiveness of radiation in inducing apoptosis in HepG2 cells, with an up-regulation in the expression of caspase-3 and Bax, a down-regulation in the expression of Bcl-2, induced DNA damage, and a decrease in the levels of CAT, SOD, and total GSH . Similarly, citrate-coated nanosilver (15.26 nm) was successful in inducing cell death in U251 cells and sensitizing the cells to radiation treatment. *In vivo* experiments using female BALB/c nude mice with U251 glioma tumors indicated that the mice treated with nanosilver via intratumoral

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injection lived longer than the control mice, followed by the mice treated with radiation alone, and finally by the mice treated with a combination of radiation and nanosilver who survived for the longest length of time [64].

Nanosilver in Combination with Other Drug Treatments

Lin Huang *et al.* [16] reported that nanosilver treatment in combination with autophagy inhibition increased the cell death and apoptosis seen in HeLa cells with nanosilver treatment, and that the autophagy induced by nanosilver treatment aided the HeLa cells to survive. This was further tested in mice by subcutaneously injecting mouse B16 melanoma cells into male C57BL/6 mice. After five days to allow the tumor to establish, the mice were treated with saline, wortmannin (an autophagy inhibitor), nanosilver, or nanosilver with wortmannin via subcutaneous injection into the tumor once a day for 8 days. Wortmannin treatment by itself did not have any effect on tumor growth, however, nanosilver treatment decreased the weight of the tumor by 42.10%, and nanosilver with wortmannin treatment decreased the tumor weight by 60.91%. To confirm the increased cell death, the tumor tissue was examined with the TUNEL assay, which indicated that the tumors treated with wortmannin and nanosilver had the most apoptotic cells followed by the tumors treated with nanosilver alone [16]. Treatment of A2780 cells with a combination of nanosilver (18 nm) and salinomycin, a monocarboxylic ionophore effective against the growth of cancer stem cells, both at their EC25 concentrations, significantly increased cell death to 81%. The proteins p53 and p21 were both upregulated by nanosilver treatment, and a combination of nanosilver plus salinomycin had an additive effect. The TUNEL assay indicated a significant increase in DNA fragmentation with the nanosilver and salinomycin treatments, and especially with the combined treatment [71]. A nanocomposite made with nanosilver dispersed on graphene

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sheets had greater toxicity of than just the nanosilver in A2780 cells and HeLa cells, and co-treatment of the cancer cells with the common chemotherapy drug cisplatin and the nanosilver-nanocomposite further increased toxicity in the cells [86,185].

Interactions With or Effects on Other Pathways

Nanosilver and the Cell Cycle:

DNA damage in cells can disrupt the cell cycle causing an accumulation of cells halted in one of the cell cycle phases: gap 1 (G1), DNA synthesis (S), or the gap 2 (G2)/mitosis (M) phase. An accumulation of cells in the subG1 phase is indicative of apoptotic cells, potentially due to irreversible DNA damage. Nanosilver (starch-capped, 6-20 nm) treatment resulted in cell cycle arrest in the G2/M phase, with cancerous human glioblastoma (U251) cells being more affected than non-cancerous IMR-90 cells. Cell cycle arrest also occurred in the G2/M phase in HK-2 cells treated with higher nanosilver (7.5 nm) treatment concentrations which appeared to result in less than 70% cell viability [77]. Cyclin-dependent kinase 1 (CDK1, also called cell division cycle protein 2 homolog (CDC2)) is important for cell cycle progression from the G2 to the M phase, and with nanosilver treatment more CDC2 was in its phosphorylated and inactive form. The enzyme responsible for activating CDC2, CDC25, was itself also more phosphorylated and inactive. As well, the protein expression levels of G2/mitotic-specific cyclin-B1 (cycline B1) were also decreased. The protein expression levels of p53 and p21 were both increased, which are involved in cell cycle arrest due to DNA damage, and the presence of DNA damage was confirmed with the cytokinesis-block micronucleus (CBMN) assay. These effects were increased by Nrf2 knockdown. Pretreatment with the antioxidant NAC alleviated the observed cell cycle arrest, DNA damage, increase in ROS; while pretreatment with L-buthionine-[S,R]-sulfoximine (BSO), which

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inhibits the synthesis of GSH, exacerbated the effects. Together, this exemplified the importance of the antioxidant response involving Nrf2 in protecting the cells from the DNA damage and cell cycle arrest induced by nanosilver treatment [77]. Treatment of human embryonic stem cell-derived neural stem/progenitor cells (hESC-derived NPCs) with 13.3 nm citrate-coated nanosilver resulted in an increase in apoptotic cells in the sub-G1 phase with both non-toxic as well as toxic nanosilver treatment. Increased DNA fragmentation was also seen using an ELISA assay confirming apoptotic cell death [3].

The Effects of Nanosilver on DNA Polymerase and Transcription:

Wang *et al*. [11] found that nanosilver can bind directly to DNA polymerase and decrease its activity in MEL cells. These erythroid progenitor cells highly express hemoglobin under normal circumstances, however, non-toxic nanosilver treatment with both spherical (10, 25, 40, and 110 nm) and plate-like (45 nm) PVP-coated nanosilver resulted in decreased mRNA expression of both α-globin and β-globin. Interestingly, the intracellular iron levels which are vital for hemoglobin production were not affected by the nanosilver treatment, and thus was not the reason for the decrease. Non-toxic nanosilver treatment (PVP-coated, 25 nm) caused silver to be accumulated in the nucleus of the MEL cells, directly interacted with RNA polymerase but not with the DNA as seen via pull down and immunoprecipitation assays, decreased RNA polymerase transcription, and decreased the amount of total RNA synthesized by over 30%. This same mechanism of suppressed RNA polymerase activity and suppressed transcription also occurs in *E. coli* cells treated with non-lethal concentrations of nanosilver and contributes to the antibacterial activity that is caused by the nanosilver. In both the MEL and *E. coli* cells, this mechanism is not a result of the released Ag^+ ions from the nanosilver, but is a silver nanoparticle specific effect. The treatment of male and

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female BALB/c mice with nanosilver via intraperitoneal injection before pregnancy resulted in embryos with a pale and anemic appearance, reduced hemoglobin in the blood, and significantly reduced growth and development. Gene expression in the embryonic liver tissue was greatly affected with the upregulation of 37 genes and the downregulation of 264 genes, with many of the downregulated genes being involved in erythropoiesis [11].

The Effects of Nanosilver on Pathways Involving Nrf2 and the Antioxidant Response:

Nrf2 is a transcription factor that is integral to the antioxidant response, and induces various xenobiotic-metabolizing and antioxidant enzymes through the electrophile response element/antioxidant response element (EpRE/ARE). When not stimulated, Nrf2 is bound to kelch-like ECH-associated protein 1 (Keap1) and Cullin-3 (Cul3) ubiquitin ligase in the cytosol where it is degraded through ubiquitination and subsequent proteasomal degradation. Nrf2 is activated as a protective response to various stressors such as ROS or electrophiles. Cysteine modifications on C151, C273, and C288 in Keap1 cause Nrf2 to be released and allow it to translocate to the nucleus where it binds to the antioxidant response element (ARE) along with small Maf proteins. This activates the expression of genes involved in the antioxidant response such as HO-1, NQO1, glutathione S transferases (GSTs) and many others [73,195,196].

Reporter gene assays for the Nrf2/ARE pathway have shown an increase in gene activation with nanosilver treatment. Prasad *et al.* [47] treated stable luciferase-reporter HepG2 cell lines for Nrf2/ARE, NFκB, and AP1 with 10 and 75 nm, citrate and PVP-coated nanosilver, and found that the Nrf2/ARE pathway was activated the strongest out of the pathways tested [47]. N27 neurons transfected with Nrf2/ARE reporter gene also resulted in activation of the Nrf2/ARE pathway with

nanosilver (10 and 75 nm, PVP-coated) treatment, and the expression of oxidative stress related genes HO-1 and NQO1 also increased [67]. DNA microarray data indicated that genes involved in the Nrf2 oxidative stress response pathway were up-regulate in HepG2 cells treated with non-toxic nanosilver (20 and 50 nm) treatments [55]. Oxidative stress was induced and the protein levels of Nrf2 and HO-1 were greatly elevated in K562 cells when treated with non-toxic levels of 27 nm PVP-coated nanosilver [73]. The up-regulation of the lncRNA ODRUL as a response to nanosilver exposure is potentially controlled by Nrf2, since pre-treatment with the antioxidant NAC decreased the nanosilver induced ODRUL up-regulation by more than half, and Nrf2 knockdown completely inhibited the ODRUL up-regulation in the K562 cells. Once up-regulated by the nanosilver treatment, ODRUL physically interacts with phosphatidylinositol 4-kinase alpha (PI4Kα), as was seen through RNA-protein pull down experiments, and regulates the subsequent PI4Kα, AKT, JNK, and Bcl-2 signalling [73]. On the other hand, in human Chang liver cells, nanosilver (5-10 nm) treatment resulted in decreased protein expression of phosphorylated and active AKT and ERK1/2. AKT and ERK are both involved in the regulation of Nrf2, and decreased AKT and ERK resulted in decreased Nrf2 protein expression in this study. With nanosilver treatment, the decreased Nrf2 did not bind to the OGG1 gene promotor, the expression of OGG1 was decreased, and thus the levels of the DNA oxidation marker, 8-oxoguanine, were not repaired and increased in the cells [173].

Nanosilver and the Insulin Signalling Pathway:

In the insulin signalling pathway, insulin binds to the insulin receptor tyrosine kinase, which activates insulin receptor substrate 1 (IRS-1) by phosphorylation, leading to activation of AKT and mTOR. In the liver, AKT regulates glucose metabolism and is involved in inducing glycogen

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synthesis via the phosphorylation and inhibition of glycogen synthase kinase-3 beta (GSK3β). The levels of phosphorylated AKT increased in the livers of male Sprague Dawley rats treated orally with up to 100mg/kg/day nanosilver (PVP-coated, 20-30 nm), as did the levels of phosphorylated IRS-1, phosphorylated GSK3β, and phosphorylated mTOR [69].

Nanosilver Effects on Copper Homeodynamics:

Copper is an essential micronutrient in the human body. Whether nanosilver has any effects on copper homeostasis in human cells or in the human body is currently unexplored. However, non-toxic nanosilver treatment of *Drosophila melanogaster* with citrate-coated nanosilver (1-50 nm) has been found to result in cuticular demelanization and to detrimentally effected their ability to move, a symptom that is also seen with copper deficiency [18]. Additionally, the activities of copper dependent enzymes such as tyrosinase and copper/zinc superoxide dismutase (Cu/ZnSOD) were decreased, with tyrosinase being involved in melanin synthesis. The activity of manganese dependent SOD (MnSOD) was not affected, indicating a specific effect on intracellular copper levels. Nanosilver was also found to interact with the copper transporters (CTR). Thus the nanosilver appears to competitively compete with the copper ions for uptake by the transporters, resulting in a lower amount of copper being brought into the cell and cellular copper depletion [18,21]. A related study done by Ilyechova *et al.* [37] examined the effects of Ag^{+} ions on albino rats, using AgCl mixed into the food. Copper deficiency was observed in the rats, as well as decreased mRNA expression of several genes such as the cuproenzyme cytochrome c oxidase subunit 4 isoform 1 (Cox4i1), and genes involved in copper homeodynamics such as Cu(I)/Ag(I)-transporter 1 (CTR1), Cu(I)/Ag(I)-transporter 2 (CTR2), Cu-chaperone for SOD1 (CCS), MT1A,

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and Cu(II) binding cytosol protein (Commd1); although the protein expression of MT1A and Commd1 did not decrease [37].

Conclusions

Nanosilver is generally taken up into cells through endocytosis, with the smaller sized nanosilver being taken up into the cells, interacting with the cellular components, and binding to biomolecules through their sulfhydryl groups. Larger sized nanosilver may be too large to be internalized, and may remain on the outside where it can trigger various receptor-mediated signalling mechanisms or cause lipid peroxidation. Inside the cell, it has generally been accepted that nanosilver produces ROS and oxidative stress in the cells, although this has been questioned in some studies where no increase in ROS was seen. This may be due to different experimental conditions or due to problems detecting the actual levels ROS with H_2DCFDA, the main dye that is used to detect ROS. Nanosilver activates the immune response and increases inflammation, with this having a beneficial effect and shortening the time required for wound healing. There are currently only a small number of studies that have been done on the effects of nanosilver on hypoxic stress, with the results currently being inconclusive, thus requiring additional research. High dose nanosilver treatment around the EC_{50} value for the cells leads to increased contact and signaling between the ER and the mitochondria, increased transfer of calcium from the ER to the mitochondrial, mitochondrial dysfunction, decrease in ATP production, and mitochondria-mediated apoptosis. It has been found that low dose nanosilver exposure can disrupt ER homeostasis and cause the induction of the ER stress response without cell death. High dose nanosilver exposure, with treatments around the EC_{50} value for the cells or higher, leads to an induction of the ER stress response and cell death. The general consensus of the studies to date is that nanosilver treatment

inhibits angiogenesis. Epigenetic changes in terms of increased DNA methylation, various histone tail modifications, and changes in non-coding RNA expression were seen. In terms of genotoxicity, DNA damage, DNA base oxidation, DNA adducts, DNA strand breaks, and chromosomal aberrations have all been observed; however, not all studies observe this, and this effect may not be permanent once the nanosilver treatment is removed. Finally, cancer cells are consistently more susceptible to nanosilver treatment than non-cancer cells, and *in vivo* studies indicate that nanosilver is an effective and promising anti-cancer agent, with no adverse effects observed in the treated animals. This anti-cancer effect can be further improved by using the nanosilver treatments in combination with other cancer treatments.

Thus, in general, nanosilver in reasonable doses has many beneficial effects and applications, especially in its anti-bacterial, anti-viral, anti-fungal, anti-cancer, and wound healing functions; while at the same time not causing adverse effects *in vivo*. As with any compound, high enough doses will induce toxicity, especially in *in vitro*.

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