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Effect of Silver and Cadmium Nanoparticles on Endocytosis and Protein Recycling in Yeast

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**EFFECT OF SILVER AND CADMIUM NANOPARTICLES ON ENDOCYTOSIS AND
PROTEIN RECYCLING IN YEAST**

A Master's Thesis
Presented to
The Graduate College of
Missouri State University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science, Biology

By
Lakshmi Sravya Rallabandi
May 2020

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EFFECT OF SILVER AND CADMIUM NANOPARTICLES ON ENDOCYTOSIS AND PROTEIN RECYCLING IN YEAST

Biology

Missouri State University, May 2020

Master of Science

Lakshmi Sravya Rallabandi

ABSTRACT

Endocytosis is a highly regulated process crucial for recycling of plasma membrane proteins and lipids. Derailments in this trafficking pathway pose threat to normal functioning of somatic cells. Engineered nanoparticles are used extensively in industry due to unique physicochemical properties. However, these nanoparticles, at high concentrations are known to create toxic effects on biological tissues. With this recent information, I investigated the potential toxicity of silver nanoparticles (AgNP) and cadmium selenium/zinc sulfide (CdSe/ZnS) quantum dots (QDs) on liquid phase endocytosis pathway in *Saccharomyces cerevisiae*. My data provided evidence that treatment of yeast cells with AgNP and CdSe/ZnS QDs resulted in FM4-64 transit defects with a significant delay of its trafficking to the final destination. To test the potential negative effects of these nanoparticles on intracellular trafficking pathways, I examined the distribution pattern of Vps10-GFP and GFP-Snc1. Both AgNP and CdSe/ZnS QDs disrupted the intracellular trafficking pathways as shown in abnormal distribution patterns of Vps10-GFP and GFP-Snc1. In addition, cells treated with cadmium sulphate and silver nitrate displayed severe defects both in endocytosis and the trafficking toward the Golgi. In summary, my study provided evidence that AgNP and CdSe/ZnS QDs affect endocytosis and endosome-Golgi trafficking. Future research would involve illustrating the molecular mechanisms behind the cell toxicity.

KEYWORDS: Endocytosis, Protein recycling, FM4-64, Silver nanoparticles, Cadmium selenium, Quantum dots, Yeast, Vps10, Snc1

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May 2020

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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I dedicate this thesis to my family.

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INTRODUCTION

Overview of Intracellular Trafficking and Endocytosis

Intracellular trafficking is a highly regulated process and involves transport of several proteins and lipid components in the form of membrane bound vesicles (Dubuke & Munson, 2016). The newly formed proteins from the endoplasmic reticulum move to the Golgi apparatus where some of these protein cargos are sorted into exocytic vesicles that are transported to plasma membrane or secreted, and others targeted to the vacuole either directly or through endosomes (Feyder et al., 2015). Endocytosis is another vesicular trafficking pathway essential for recycling of cellular surface receptors and lipids, which is pivotal for the maintenance of cellular homeostasis (Smaczynska-de Rooij et al., 2010). Endocytosis takes place when a portion of the plasma membrane invaginates and forms into a bud like structure. This bud like structure later gets separated from the membrane and forms a vesicle. This process occurs with the help of protein complexes (Idrissi & Geli, 2014). Nearly 50 proteins are transiently involved in this process in budding yeast (Goode et al., 2014). Any soluble materials from the outside environment can also be internalized through the same mechanism (Riezman, 1985). Likewise, nanoparticle entry into yeast cells occurs through this route. Results reported in the literature have shown that the budding yeast *Saccharomyces cerevisiae* is an excellent eukaryotic model to study protein machinery modulating the endocytosis pathway (Feyder et al., 2015). Ease in culturing, genetic manipulation, and similarity to mammalian cells are the factors contributing to its use (Laidlaw & MacDonald, 2018).

General Information on Engineered Nanoparticles

Engineered nanoparticles are defined as particles smaller than 100 nm in any dimension and exist either in an unbound state or as an aggregate (Márquez et al., 2018). These particles have unique physiochemical properties such as large surface area, shell coating, crystalline structure, and strong reactivity (Liu & Tang, 2019; Sukhanova et al., 2018). Hence, they are widely used in several industrial products such as cosmetics, toothpastes, and food packaging. In addition to commercial products, nanoparticles are used in nanomedicine for imaging and drug delivery. These applications are due to their ability to cross biological barriers and enter the cells (Foroozandeh & Aziz, 2018). However, this translocation poses a threat to human health. Different factors govern the entry of nanoparticles into the cellular system. Nanoparticle coating is one of them. Depending on whether the nanoparticles are hydrophilic or hydrophobic, entry of nanoparticles into the cells is different. In particular, hydrophilic nanoparticles cannot readily pass through the plasma membrane, and therefore, they are internalized via endocytosis (Zhao et al., 2011). Size, shape, dispersion, cell density, cell type, solubility, and charge are other factors regulating nanoparticle entry (Kettiger et al., 2013). Nevertheless, in cells lacking endocytic machinery, nanoparticle entry can occur through passive transport (Kou et al., 2013; Vácha et al., 2011).

Nanoparticles interact with different intracellular proteins. Their binding can either enhance, decrease, or show no effect on protein functionality and stability. Some nanoparticles also cause protein aggregations which could further lead to neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (Fröhlich, 2012). These potential harmful properties support continued studies on the pathological effects of nanoparticles on human health.

Nanomaterial Properties and Toxicity

Size: Nanoparticle size has a crucial role in determining its internalization kinetics and subcellular distribution (Shang et al., 2014). It was found that small size nanoparticles are transported more efficiently when compared to larger ones (Kettiger et al., 2013). The optimal size for efficient uptake was found to be 50 nm in size (Foroozandeh & Aziz, 2018). In addition, size of nanoparticle dictates the mode of entry. It was found that nanoparticles of size 1-10 nm enter the cell through passive diffusion, and 10-100 nm size particles enter through caveola mediated endocytosis. Particles greater than 100 nm gain entry via macro pinocytosis or pinocytosis (Panzarini et al., 2018). It was also noted that some nanoparticles smaller than 25 nm can gain entry and reach the perinuclear space by other pathways such as non-clathrin, non-caveolae mediated and cholesterol independent endocytosis (Foroozandeh & Aziz, 2018). Other nanoparticles such as quantum dots and nanoclusters less than 10 nm in size accumulate at the plasma membrane prior to internalization (Shang et al., 2014). Following the internalization, nanoparticles are either subjected to the endo-lysosomal degradation pathway or localized in the cytosol (Kettiger et al., 2013)

Shape of Nanoparticles: In a study done using gold nanoparticles, it was shown that spherical shaped nanoparticles are more readily taken up by the cellular system than the rod-shaped particles. This was possibly due to longer wrapping time for rod shaped particles to be internalized into the membrane structure (Champion et al., 2007; Kettiger et al., 2013; Zhao et al., 2011). Spherical shape particles are spontaneously taken up by lipid membranes (Spangler et al., 2016). Rod shaped nanoparticles gain entry into the cell either through caveolae or clathrin-mediated endocytosis, and other forms of nanoparticles such as triangular or star shape are through clathrin- mediated endocytosis (Panzarini et al., 2018).

Charge: Surface charge is one of the essential factors in cellular entry and intracellular interactions. Depending on the surface charge, nanoparticles can be neutral, negatively charged, or positively charged. Positively charged nanoparticles enter paracellularly, through macropinocytosis and clathrin-mediated pathways (Foroozandeh & Aziz, 2018; Panzarini et al., 2018). These particles readily adsorb to proteins and have the ability to disrupt the plasma membrane integrity, thereby increasing the fluid mechanics (Kettiger et al., 2013). In addition to electrostatic interactions with the cell membrane, the positively charged particles interact with DNA due to its negatively charged nature and prolong the G0/G1 phase of the cell cycle (Liu et al., 2011). Neutral nanoparticles are endocytosed through the caveolae-mediated pathway, clathrin, or caveolae independent pathways (Kettiger et al., 2013). Negatively charged particles enter via clathrin mediated endocytosis (Brandenberger et al., 2010). Negatively charged nanoparticles result in intracellular damage although, this mechanism isn't clear (Fröhlich, 2012).

Toxicity: Size, shape, material, surface charge, concentration, and hydrophobicity are factors that influence the cytotoxicity of nanoparticles (Fröhlich, 2012; Panzarini et al., 2018). The mode of entry of nanoparticles and their intracellular location also dictate the toxicity (Foroozandeh & Aziz, 2018). Small size nanoparticle cause greater cytotoxicity due to a greater surface area relative to their mass (Shang et al., 2014). In a study done on gold nanoparticles ranging from 0.8 to 15 nm, it was noted that 15 nm size particles were 60 times less toxic than 1.4 nm for different cell types (Sukhanova et al., 2018). Needle shaped, fiber, and plate shaped particles have higher reactivity and cause more toxicity than rod or spherical shaped particles (Fröhlich, 2012; Sukhanova et al., 2018). Positively charged nanoparticles induce higher toxicity and cause cell death in comparison to other charges as they are taken up readily by the cell

membrane (Foroozandeh & Aziz, 2018). For instance, metal ions like silver and cadmium cause significant cell damage (Sukhanova et al., 2018).

Specific Properties of Silver Nanoparticles

Silver nanoparticles (AgNPs) are one of the most commonly used engineered nanoparticles with applications in consumer products and nanomedicine (Durán et al., 2015; Márquez et al., 2018). Of the 1,300 marketed nanoparticle products, 25 percent of them contain silver (Rejeski, 2011). AgNPs are used in several biomedical applications such as wound dressings, catheters, bone cement, and cardiac valves due to their excellent anti-microbial properties (Roe et al., 2008; Wu et al., 2019). In addition, AgNPs are effective in environmental treatments such as waste-water management (Panzarini et al., 2018). Despite the wide applications of nanoparticles in several industries, they may cause deleterious effects on human health and the environment at high concentrations. AgNPs have been shown to cause changes in cellular structures and cause gene alterations (Durán et al., 2015).

Small size AgNPs penetrate the nuclear pore, generate reactive oxygen species, cause DNA damage, cell cycle arrest, and mitochondrial dysfunction (Panzarini et al., 2018). In an experimental study done on mice by intravenously injecting AgNPs of various sizes, it was observed that toxicity caused by large size particles was less than small size particles due to greater surface area, penetration ability, and concentrated release of Ag ions into the cell culture (Sukhanova et al., 2018). Another experiment done to study genotoxicity of AgNPs of 5 mg/kg on mice revealed that these nanoparticles lead to DNA breakage and cause oxidation of purine bases in tissues (Asare et al., 2016). Four pathways have been suggested for the AgNP-induced cell death, including necrotic pathway by rupture of the plasma membrane, mitochondria

dependent cell death, and ER-mediated and lysosomal-mediated apoptosis (AshaRani et al., 2009; Panzarini et al., 2018)

AgNPs act on various cellular components and cause cytotoxicity, genotoxicity, and cell cycle arrest (Singh & Ramarao, 2012). These effects are either by apoptosis and necrosis or by generation of reactive oxygen species, and this has been proven experimentally in simple salt solutions and cell culture media (Durán et al., 2015; Singh & Ramarao, 2012).

These nanoparticles are internalized at an optimal range of 30-50 nm in different cell culture lines (Wu et al., 2019). AgNP around 5 nm employ caveolae-mediated, and about 20 nm size particles are taken up by clathrin-mediated endocytosis. Larger particles around 50 nm use three different pathways, but caveolae-mediated pathway is most common among them. Finally, 100 nm size particles are internalized via clathrin mediated pathway and micropinocytosis (Wu et al., 2019). The different routes of entry into cellular system are due to AgNP aggregation, meaning that the smaller nanoparticles come together to form larger sizes in biological media (Lankoff et al., 2012).

Specific Properties of CdSe/ZnS Coated Quantum Dots

Semi-conductor nanoparticles such as cadmium, selenium have size dependent properties and exhibit strong photoluminescence and optical properties (Biju et al., 2008). Therefore, these particles are widely used in light sensors, optoelectronic devices, and chemical libraries (Suresh & Arunseshan, 2014)

Quantum dots are structurally made up of a crystalline core that can be metal, or semiconductor encased in a shell. Cadmium and selenium are two popularly used quantum dot constituent metals and cause toxic effects on humans (Biju et al., 2008; Hamilton, 2004; Henson

& Chedrese, 2004; Satarug & Moore, 2004). These metals also have the ability to cross the blood brain barrier (Hardman, 2006). The cap or shell can be made of different metals and belong to different group series. For example, ZnS coat is categorized under group II – IV series of quantum dots (Hardman, 2006). The presence of capping structure reduces the leakage and enhances physical stability of quantum dots thus making them useful in drug delivery (Biju et al., 2008; Hardman, 2006).

Role of FM-4-64 in Vacuolar Staining

FM-4-64 is a lipophilic styryl dye that binds plasma membrane lipids and follows the liquid phase endocytosis and finally localizes to the vacuolar membrane in yeast (Zheng et al., 1998). Though a major fraction of the plasma membrane-bound dye is recycled back to the culture media, a minor fraction of it finally arrives at the vacuolar membrane. This property makes FM4-64 efficient in tracing fluid phase endocytosis in yeast cells by labeling the membranes of the pathway and delivering to the vacuole (Gurunathan et al., 2000).

In a study done by Vida et al., 1995, it was found that FM-4-64 staining activity was time, temperature, and energy dependent. They found that when FM-4-64 dye was used at different temperature and time intervals, the staining varied. At 0°C, plasma membrane staining was noted and when cells were warmed to 25°C, plasma membrane staining decreased. The time interval was crucial for endosome and vacuolar staining, which were evident at 5-10 mins and 60 mins respectively (Vida & Emr, 1995). These properties make FM-4-64 an excellent endocytic marker for my study.

Role of Vps10

Vps10 (Vacuolar protein sorting 10) is a type 1 transmembrane protein known to play a significant role in intracellular trafficking in eukaryotic cells by sorting acid hydrolases to the vacuole(Bonifacino & Rojas, 2006; Marcusson et al., 1994).This protein is presumed to be a key component in the etiology of neurodegenerative diseases given its role in endosomal sorting and the retrograde pathway(Lane et al., 2012). Vps10 is also required for efficient functioning of the yeast vacuole (Lane et al., 2012)

Role of Snc1

Snc1, is a V snare and part of multiple transport mechanisms that are constantly recycled between the plasma membrane and the trans Golgi network through endosomes (Robinson et al., 2006). In yeast cells, these membrane proteins are localized to post Golgi transport vesicles and are required for normal secretion (Couve & Gerst,1994). In a study done by Couve &Gerst,1994 it was found that yeast cells lacking Snc1 membrane protein accumulated transport vesicles and also defective vesicle fusion suggesting the pivotal role of this protein in post Golgi vesicle transport (Couve & Gerst,1994). In wild type cells, Snc1-GFP shows plasma membrane staining on budding cells (Robinson et al., 2006).

Problem Statement and Hypothesis

Ever since the advent of nanomedicine, benefits have been given prime importance. Although, researchers have focused on toxicity of nanoparticles and the effects of these particles on human health, there still exists a need to study the effect within the biological pathways.

Findings from prior investigations have shown that artificial nanoparticles cause cellular alterations. In a study done by Jiang et al., 2008, it was found that nanoparticles within the range of 2-100 nm alter the basic cell functions. They also affect the signaling pathways when in range of 40-50 nm (Jiang et al., 2008). In another study, it was found that AgNPs of 4 nm induce higher levels of reactive oxygen species when compared to larger AgNPs, with other characteristics remaining the same (Shang et al., 2014). In addition, a study on AgNPs demonstrated by a growth assay the inhibitory effect of these particles on yeast growth at concentrations of 5 µg/mL or above. This was complemented by RNA seq data that showed genes for cell wall, plasma membrane, vesicular trafficking, and mitochondrial integrity were all downregulated (Horstmann, Campbell, et al., 2019).

Furthermore, Horstmann and coworkers recently revealed that CdSe/ZnS quantum dots (QDs) also negatively affect the expression of several genes functioning for endocytosis and the vesicular transport toward the Golgi, which prompted me to determine the effect of AgNPs and cadmium selenide (CdSe/ZnS) QDs on endocytosis and endosome-to-Golgi trafficking. This study will identify the defects in these trafficking pathways, and the results obtained from the proposed research will provide insights into understanding the impact on membrane trafficking induced by manufactured nanoparticles. I hypothesized that the treatment with these nanoparticles made from silver and cadmium will have an effect on these pathways due to release of corresponding ions.

My study had two major goals. Firstly, to investigate the effect of AgNPs and CdSe/ZnS QDs on liquid phase endocytosis in yeast using FM4-64 dye. Next, I wanted to study the effect of these nanomaterials on protein recycling, specifically endosome-to Golgi trafficking. For this study, I used cell staining and confocal microscopy techniques.

MATERIALS AND METHODS

Strains Used in This Study

All strains listed in Table 1 were grown on nutrient rich media plates for about 3 days at 30°C and then grown in specific liquid cultures. I used yeast extract peptone dextrose (YPD) media for Wildtype (WT) cells. KKY 1886 (WT, Vps10-GFP) and KKY 1525 (WT, GFP-Snc1) strains were grown on media lacking leucine. All the liquid cultures were grown 16-18 hours in their corresponding nutrient media at 30°C in a shaking incubator.

Nanomaterials Used for This Study

Spherical shape silver nanoparticles (20 nm) dissolved in 2 mM sodium citrate solution (pH 7.6) at a concentration of 20 µg/mL, LOT # SDM0049 were obtained from Nano-Composix (San Diego, CA). This product has a light absorption range of 395- 515 nm. Cadmium selenide/ZnS quantum dots of 5.0-9.5 nm size (CdSe/ZnS QDs, yellow), Product# CZW-Y-25, LOT # LW059915K25302 were from NN-Labs, (Fayetteville, AR) . This product has a light excitation range of 500-640 nm and emission range of 515-660 nm

Cell Culture and Treatment with Nanoparticles

All the strains used for this study (Table 1) were treated with 375 µL of silver nanoparticles and 7.5 µL of CdSe/ZnS QDs ensuring the final concentration to be 5 µg/mL. Positive control groups were treated with 0.75 µL silver nitrate (5 µg/mL), Product # 701116, purchased from Fisher Scientific, Inc (Hampton, NH). The cadmium sulphate stock solution was

prepared by suspending cadmium sulphate powder, purchased from Fisher Scientific, Inc (Hampton, NH) in water resulting in a final concentration of 100 µg/mL. Seventy-five µL of this cadmium sulphate solution was used to treat all yeast strains with a final concentration of 5 µg/mL in liquid culture.

All strains were grown in the above-mentioned appropriate media at 30°C overnight. The optical density (OD) of the culture medium was measured at 600nm using a BioMate 3S spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The culture medium was then diluted to an OD of 0.4. Following that, the culture medium was treated with nanoparticles and incubated at 30°C for 1.5 hr until the culture reached an OD at 600 nm of 0.5-0.6. The treated culture volume was 1.5 mL. Every yeast culture used in the experimental design was treated with nanoparticles at a final concentration of 5 µg/mL.

Assessing FM4-64 Transit from the Plasma Membrane to the Vacuole

To investigate the potential impact of AgNPs and CdSe/ZnS QDs on the liquid phase endocytosis, FM4-64 dye was used. As stated, FM4-64 marks the transit from the plasma membrane to the vacuole.

One control group was not treated with any nanoparticles, while the positive control groups were incubated with silver nitrate or cadmium sulphate. Experimental groups were grown with the indicated amount of AgNPs or CdSe/ZnS QDs.

After a 90 min incubation with the nanoparticles at 30°C in a shaker, the cell cultures were centrifuged for 10 mins at 1000 rpm at 4°C in an Eppendorf tabletop centrifuge (Model No.5418). Then, the resulting cell pellet was resuspended with 50 µL of ice-cold Synthetic

Defined (SD) Complete Media (100 mL), which contains Complete Supplement Mixture-0.67 g, glucose-2 g, Yeast nitrogen base-0.17 g, and water up to 100 mL

Next, the cell suspension was incubated with FM4-64 (Thermo fisher Scientific, Cat No T3166) at a final concentration of 300 μ M (6 μ L of 1 mM FM4-64 per 50 μ L cell suspension) for 15 minutes on ice, followed by incubation at 30°C in a shaker for 30 mins. The cell suspension was immediately centrifuged at 4°C for 1 min in Eppendorf tabletop centrifuge. The supernatant was removed, and the cells were subjected to two wash periods for 1 min at 4°C each with 50 μ L and resuspended in 1 mL SD complete media. Cells were harvested, transferred to a glass slide, and covered with a coverslip. The cells were kept on ice until imaging and were visualized using a Leica DMI8 confocal microscope within 10 mins after removing from ice. The FM4-64 stained cells were visualized using rhodamine channel with an exposure time of 200 ms and magnification of 63x oil immersion.

Fluorescence Microscopy

The wild type cells expressing Vps10-GFP and GFP-Snc1 were treated with 5 μ g/mL of nanoparticles as indicated above. The resulting culture was centrifuged in an Eppendorf tabletop centrifuge (Model No. 5418) for 10 minutes at 1000 rpm. The cells were then placed on a glass slide and covered with a coverslip for visualization using a Leica DMI8 confocal microscope. Single channel exposure was used with an exposure time of 200 ms and visualized at 63X magnification and an oil immersion. GFP laser was used to visualize the cells.

Statistical Analysis

All data from the microscopy was replicated in three individual sets with each set including 30-50 budding cells ensuring the total count of cells didn't exceed 150 cells. From the 30 min FM4-64 chase experiment, cells showing ring-like vacuolar membrane selectively stained by FM4-64 were categorized into the 'normal group'. In contrast, cells displaying the following FM4-64 distribution patterns were categorized into the 'abnormal group': FM4-64 puncta at or near the plasma membrane, FM4-64 fluorescence covering all areas of the plasma membrane homogenously, and dense cytoplasmic staining of FM4-64. From the Vps10-GFP distribution pattern experiment, cells showing Vps10-GFP cytoplasmic puncta were considered normal phenotype, but cells showing bright and enlarged Vps10-GFP puncta were categorized as abnormal. In addition, cells showing ring like structure with Vps10-GFP were categorized into abnormal phenotype. In GFP-Snc1 distribution experiment, cells showing polarized localization of GFP-Snc1 at the bud membrane were categorized as normal phenotype, but all the cells which show loss of polarization at the bud membrane were accounted as abnormal. All the abnormal cells were counted with the above-mentioned criteria, and then mean and standard deviation were calculated using Excel. Single factor ANOVA was done in Excel to assess the differences within and between the groups, and all experiments reported F values greater than F critical value. Next, student *t* test (2 tails, two sample was done using Excel and p values <0.05 were reported in asterisks.

RESULTS

Nanoparticles Cause FM 4-64 Transit Defects

Horstmann and coworkers recently demonstrated that AgNPs and CdSe/ZnS QDs affects expression levels of genes implicated in yeast endocytosis and the vesicular trafficking toward the Golgi (Horstmann, Campbell, et al., 2019; Horstmann, Kim, et al., 2019). I hypothesized that the treatment with these nanoparticles made from silver and cadmium will have an effect on these pathways due to release of corresponding ions.

To test the potential toxic impact of these nanoparticles on the yeast endocytic pathway, FM4-64, a compound that follows the endocytic transit from the plasma membrane to the vacuole (Zheng et al., 1998), was incubated with cells for 30 min. Non-treated control cells displayed FM4-64 fluorescence at the rim of the vacuole after a 30 min incubation, signifying that the transit is completed (Fig 1, A&B). Consistent with my hypothesis, 77.7 ± 15.5 % of AgNP-treated cells showed several FM4-64 puncta near the plasma membrane or in the cytoplasm, suggesting that FM4-64 transit rate is significantly delayed (Fig 1, A&B). In CdSe/ZnS-treated cells, 87.4 ± 5.7 percent of cells had FM4-64 fluorescence both at the plasma membrane and at the rim of the vacuole. Interestingly, cells with these Cd QDs contained more than one vacuole in each cell, suggesting a defect in homotypic vacuolar fusion (Fig 1, A). Cytoplasmic accumulation of FM4-64 dye was seen in 85.5 ± 6.6 % of silver nitrate-treated cells (Fig 1, A&B). Lastly, 83.3 ± 13.4 % of cadmium sulphate-treated cells showed a similar distribution phenotype of FM4-64 as CdSe/ZnS QDs (Fig 1, A&B).

Taken together, all nanoparticles interfered with the normal FM4-64 transit, resulting in abnormal localization of FM4-64 near the periphery of the plasma membrane. A previous study revealed that silver ions released from silver nitrate solution are 50 times higher than those released from silver nanoparticles (Sillapawattana et al., 2016). Consequently, our silver nitrate-treated cells showed a greater defects in comparison to the AgNP treatment groups (Fig1B). It is noteworthy that the effect of CdSe/ZnS QDs was higher than that caused by AgNPs as reflected by the higher percentage of abnormal cells (Fig 1B).

Vps10 Distribution Pattern Defects

Vacuolar protein sorting 10 (Vps10) is a type 1 transmembrane protein known to play a significant role in intracellular trafficking in eukaryotic cells as it shuttles back and forth between the endosome and the Golgi (Bonifacino & Rojas, 2006). As stated in the prior section regarding the alteration of trafficking genes upon treatment of AgNP and Cd QDs (Horstmann thesis, unpublished), my working hypothesis was that these nanoparticles would lead to defects in the distribution pattern of Vps10-GFP. As expected, the majority of non-treated cells (66.35 %) displayed a number of cytoplasmic Vps10-GFP puncta (Fig 2, A&B), whereas 82.2 ± 13.9 % of AgNP-treated cells, 80.1 ± 6.8 % of CdSe/ZnS QD-treated cells, and 76.45 ± 7.09 % of cells with CdSO₄ showed bright, enlarged Vps10-GFP puncta (Fig 2, A&B). Finally, 83.6 ± 0.5 % cells treated with silver nitrate showed abnormally big Vps10-GFP puncta and ring like structures, mimicking the shape of a vacuole (Fig 2, A&B).

In summary, in normal cells GFP-Vps10 showed small cytoplasmic puncta, whereas all the treated wild type cells showed bigger and brighter puncta and abnormal ring structures. Of the different nanoparticles used, silver nitrate showed greater percentage abnormality.

Snc1 Distribution Pattern Defects

Snc1 is an important constituent of multiple transport pathways and constantly recycled between the plasma membrane and *trans* Golgi network through endosomes (Robinson et al., 2006). Snc1 is a v-SNARE localized at the plasma membrane. It presents at the emerging bud, a site of secretion, and mediates fusion between secretory vesicles and the plasma membrane (Feyder et al., 2015). In wild type cells, GFP-Snc1 is accumulated at the budding cell plasma membrane (Robinson et al., 2006), indicating functional intracellular trafficking (TerBush et al., 1996). Consistently, 81.45 ± 9.51 % of non-treated cells exhibited GFP-Snc1 polarized at the bud (Fig 3, A&B).

My working hypothesis was that GFP-Snc1 in cells treated with AgNPs or CdSe/ZnS QDs would change its localization, leading to a depolarized distribution. As expected, 74.7 ± 14.7 % of cells treated with AgNPs and 60.6 ± 11.6 % of CdSe/ZnS QDs treated cells lost the polarized phenotype, respectively (Fig 3, A&B). On the other hand, following silver nitrate and cadmium sulphate treatment, 84.9 ± 10 % and 87.9 ± 3.1 % of cells showed GFP-Snc1 polarization abnormality, respectively (Fig 3, A&B)

Taken together, treatment with these nanoparticles resulted in loss of polarized phenotype of GFP-Snc1. Amongst all, silver nitrate caused the most severe polarization defects.

DISCUSSION

Engineered nanoparticles have wide applications in industry, but can have negative impacts on humans and the environment (Bottero et al., 2015; Lowry et al., 2012; Nel et al., 2006; Wan et al., 2018; C. Y. Wu, 2005).

I have chosen AgNPs and CdSe/ZnS QDs due to their immense potential in various industries. For example, CdSe/ZnS QDs are popular and widely used in cancer and tumor vasculature visualization because of their photoluminescent properties (Biju et al., 2008). AgNPs are used in drug delivery and as medical device coatings due to their antimicrobial properties (Ge et al., 2014). Naked semiconductor QDs such as CdSe are very toxic as they aggregate. In order to mitigate this, ZnS coating is present which reduces toxicity by suppressing the dissolution of cadmium ions. Nevertheless, it has been reported that a minute amount of Cd ions can be released even with the presence of the ZnS coat, and upon its release, cadmium has a half-life of 20 years in human cells (Hardman, 2006). This warrants further research on the potential impact of CdSe/ZnS QDs both in human cells and other experimental model organisms to benefit human health and the environment. In spite of substantial research on both of these nanoparticles, an important aspect of research that has not been investigated is their negative impact on cellular events like endocytosis and trafficking.

Toxic effects induced by AgNPs are size dependent and have been proven in different microbial species and mammalian cell culture lines (Ivask et al., 2014). In particular, the smaller the size of nanoparticles, the more severe is its toxicity (Recordati et al., 2016). It is mainly because smaller AgNPs are able to gain entry into the cell much more rapidly (Kim et al., 2012). For instance, a recent study done by injecting mice intraperitoneally with different AgNP sizes, such as 10, 60, and 100 nm, revealed that 10 nm AgNPs caused death of mice within 24 hours,

but the other two categories showed no symptoms (Cho et al., 2018). In addition, Cullen and coworkers (2019) found that treatment of 5 $\mu\text{g/mL}$ of 20 nm AgNPs resulted in a significant growth delay in yeast, and therefore, I chose to study the impact of this particular size of AgNP for my thesis research.

Endocytosis Defects in the Presence of AgNPs and CdSe/ZnS QDs

Endocytosis plays a crucial role in cellular homeostasis (Lv et al., 2016). It also has a role in intracellular signaling cascade regulation (Doherty & McMahon, 2009), and hence it is important that this biological pathway stays intact to ensure proper functioning of the cellular physiology. It has been widely accepted that Cd ions negatively impact the endocytic pathways. In a study with rat kidney cells, it was noted that Cd treatment resulted in a drastic endocytic delay of FITC-labeled dextran in proximal tubule cells (Herak-Kramberger et al., 1998). Cd ions also effect plant cells. In a study with *Picea wilsonii* (pine tree) using time-lapsed FM4-64 fluorescence microscopy revealed that FM4-64 dye uptake via endocytosis was drastically delayed in Cd-treated pollen tube compared with control groups, suggesting that endocytosis was disrupted in pollen tubes in response to Cd stress (Wang et al., 2014). Given the approved toxicity mediated by Cd ions, the above-described endocytic defects are not surprising. However, my results of FM4-64 transit defects caused by the incubation of yeast cells with 5 $\mu\text{g/mL}$ of CdSe/ZnS QDs, which was lower than lethal doses (Horstmann thesis, unpublished), was somewhat unexpected because the used CdSe QDs are coated with ZnS. Therefore, it may be not illogical to conjecture that a small amount of Cd ions most likely leaked into the system. Free Cd ions, if any, can interact with any negatively charged molecules such as phospholipids and affect the dynamic nature of plasma membrane, thereby possibly inhibiting the membrane invagination,

a prerequisite step for endocytosis. It is noteworthy to point out that CdSe/ZnS QD-treated yeast cells also displayed multiple small vacuoles stained by FM4-64 as well as the plasma membrane stained homogenously. As explained, FM4-64 staining of the plasma membrane can be attributed to endocytic defects influenced by the interaction of Cd ions and phospholipids. In regard to the observation of the small vacuoles in CdSe QD-treated cells, one cannot rule out the possibility that the amount of leaked Cd ions insufficient to shut down the liquid phase endocytosis completely. A fraction of FM4-64 carrying vesicles might pinch-off of the plasma membrane to follow the liquid phase endocytosis route and arrive at the rim of the vacuole. Finally, the presence of multiple smaller vacuoles in Cd QD-treated cells can reflect a defect in vacuole-to-vacuole fusion, but this interesting possibility should be explored further. It appears that the abnormal FM4-64 transit with Cd QDs in yeast is somewhat consistent with the previous study that reported a significant delay of dextran red, a liquid-phase endocytosis marker, toward the vacuole in plant cells treated with CdSe/ZnS QDs for 2 hrs (Etxeberria et al., 2006).

There is a plethora of literature devoted to illustrating the internalization of AgNPs into different cell types, while I was not successful in finding many studies addressing AgNP's impact on fluid-based endocytosis and hence we have limited knowledge. However, a group of researchers tested the impact of CdSe/ZnS on liquid-phase endocytosis by assessing the transit of FM4-64 in plant cells and found that FM4-64 dye was localized in the cytoplasm, but not at the rim of the vacuole in CdSe/ZnS-treated cells. This abnormal phenotype in the plant cell essentially mirrored the FM4-64 puncta accumulation near the plasma membrane of yeast cells with both nanoparticles. Further, lack of FM4-64 stained vacuoles in AgNP-treated yeast cells indicates that endocytic vesicles are formed, which can be concluded based off the observation of FM4-64 cytosolic puncta. However, they are not properly delivered to their final destination,

the vacuole. This might have resulted due to a defect in cytoskeletal highways responsible for endocytic vesicle delivery. Alternatively, these vesicles may not readily fuse with the final destination to unload FM4-64 at the rim of the vacuole.

In light of these findings, it is worth speculating that the significant delay in FM4-64 transit in the cells treated with AgNP or CdSe/ZnS QDs is most likely as the nanoparticle enter the cell through endocytosis and dysregulate the endocytic process.

Endosome and Golgi Trafficking Defects

Vps10 and Snc1 are transmembrane proteins that serve as excellent cargos to study the endosome-to-Golgi transport mechanism. My data showed that the distribution patterns of both Vps10-GFP and GFP-Snc1 are significantly altered upon incubation of AgNPs or CdSe/ZnS QDs with yeast cells, demonstrating their trafficking defects.

Findings from the literature have shown that in the presence of various nanoparticles, mitochondrial stress is elevated (Gupta & Gupta, 2005), which causes a decrease in ATP production. This may lead to disorganization of various cell functions such as cellular movement and intracellular trafficking (Panariti et al., 2012). For example, a study done using iron oxide NPs demonstrated that the nanoparticles dysregulated microtubules causing cell differentiation and morphology defects (Buyukhatipoglu & Clyne, 2011). In our study, we presume the nanoparticles have altered the functionality of cytoskeleton and this potentially could have led to distribution defects of Vps10 and Snc1.

In a study done using polystyrene bead nanoparticles on macrophages, it was found that these particles after entering the endosome form large vesicle like structures and prevent the recycling of proteins back to the cell surface (Xia et al., 2016). Based on these results, I predict

the loss of phenotype in GFP-Snc1 and abnormal cytoplasmic puncta in Vps10 could be a result of the derailment caused by nanoparticles.

Findings from my study provided evidence that AgNPs and CdSe/ZnS QDs are physiologically disruptive to different biological pathways, including endocytosis and the endosome-to Golgi trafficking. Furthermore, results from my study demonstrated that CdSe/ZnS QDs were more disruptive to the endocytosis process while AgNPs were more disruptive of endosome-to-Golgi trafficking.

FUTURE STUDIES AND LIMITATIONS

Future research should be directed towards studying the molecular machinery contributing to endocytosis and endosome-to-Golgi trafficking defects. As we speculated, nanoparticles might alter the functionality of cytoskeleton. In a study done on mouse chromaffin cells to study the effect of CdSe/ZnS QDs on calcium channel functionality and calcium dependent secretion, it was found that the QDs disrupted the calcium influx and also effected the functionality of exocytic machinery (Gosso et al., 2011). In light of this, it is possible that the effect of CdSe/ZnS observed on endocytosis process could potentially be due to competition of Cd^{+2} and Ca^{+2} ions. Future experiments should be directed towards analyzing this effect.

The sample size wasn't consistent throughout the study. The number of budding cells ranged from 30-50 for each individual set. However, ensuring the total count of cells didn't exceed 150 cells. Experiments were done on three different days and each included only one sample per set.

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Table 1: Yeast Strains used in this study:

Strain Number	Source	Genotype
KKY915	M. Hall	<i>Mat a leu2-3,112 ura3-52 rme1 trp1 his4 GAL4+HMLa</i>
KKY1525	(Saimani et al., 2017)	<i>MAT a his3 ΔLeu2d met15 ΔUra3 ΔGFP-Snc1-LEU</i>
KKY1886	(Makaraci et al., 2019)	<i>MAT alpha his3 ΔLeu2 ΔLys2 ΔUra3 Δ;Vps10-GFP-LEU</i>

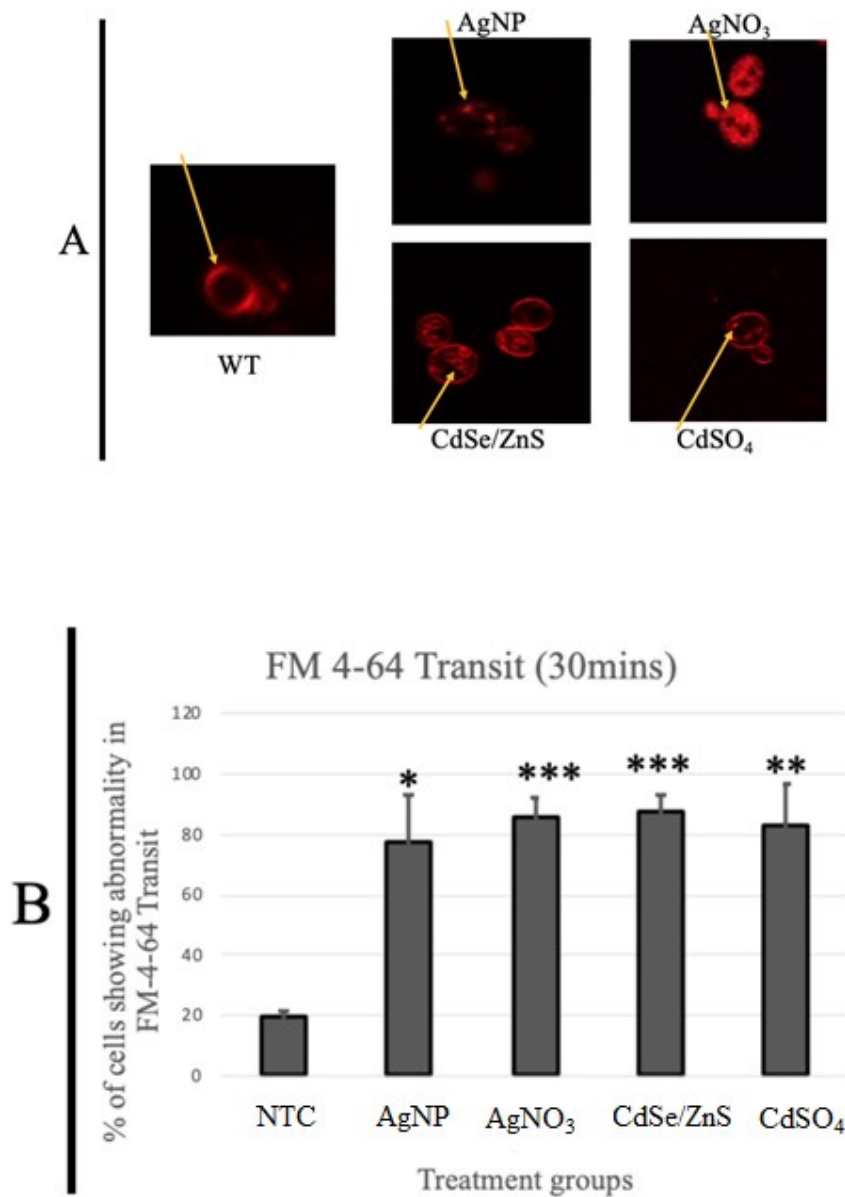


Fig 1: FM4-64 Transit Defects. **A).** Representative images of FM4-64 localization in wild-type and treated WT cells. **B).** Quantification of percentage of abnormal cells in wild type post treatment with 5 $\mu\text{g/mL}$ of AgNP, AgNO₃, CdSe/ZnS and CdSO₄ for 1.5 hr in a shaker at 30°C and then treated with 6 μL of 1 mM FM4-64, 3 individual sets with each set with one sample including 30-50 budding cells, 117 cells of AgNP, 137 cells of CdSe/ZnS, 106 cells of AgNO₃ and 117 cells of CdSO₄ ensuring the total number of cells no more than 150 cells. * represents $p < 0.05$, ** represents $p < 0.01$ and *** represents $p < 0.001$. WT- Wild Type cells and NTC- Non treated controls.

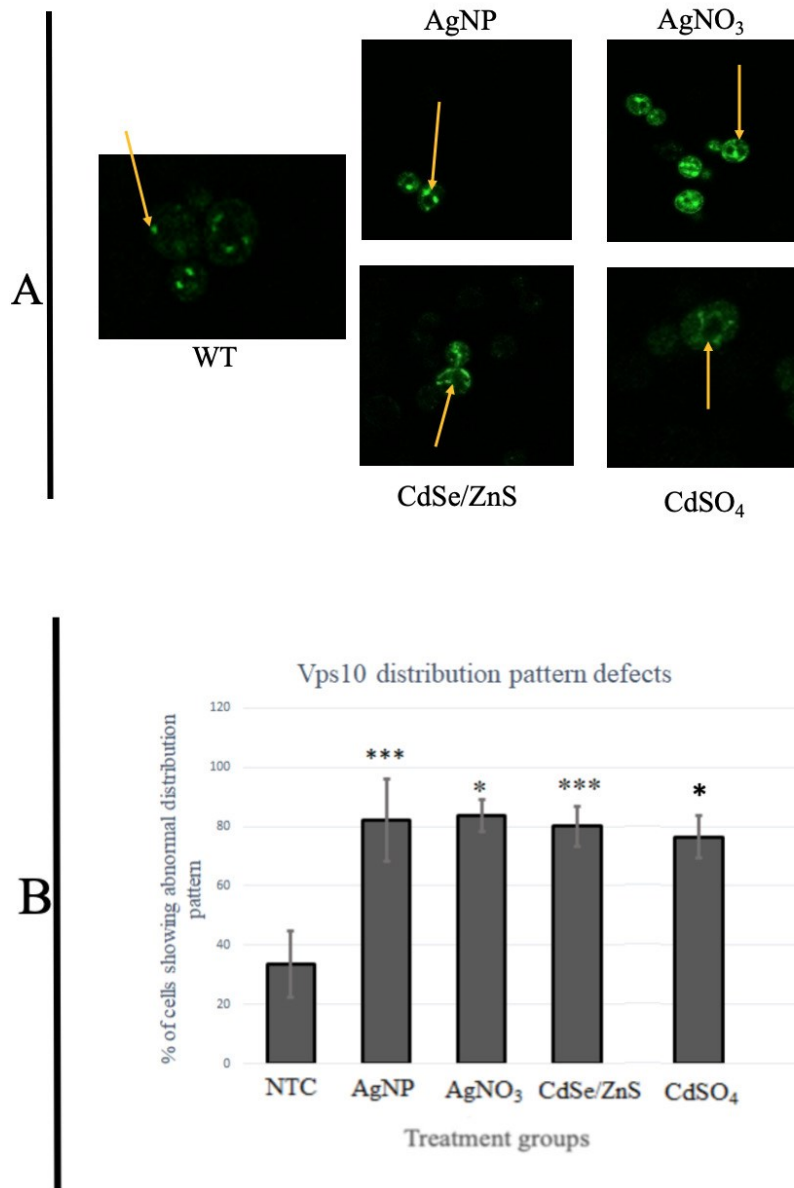


Fig 2: Vps10-GFP Distribution Pattern Defects. A). Representative images of Vps10-GFP localization in wild-type and treated WT cells. **B).** Quantification of percentage of abnormal cells in wild type following treatment with 5 $\mu\text{g/mL}$ of AgNP, AgNO₃, CdSe/ZnS and CdSO₄ for 1.5hr in a shaker at 30°C, 3 individual sets with one sample each including 30-50 budding cells ensuring the total number of cells no more than 150 cells. 112 cells of AgNP, 110 cells of CdSe/ZnS, 145 cells of AgNO₃ and 121 cells of CdSO₄. *** represents $p < 0.001$, * represents $p < 0.05$. WT- Wild Type cells and NTC- Non treated controls.

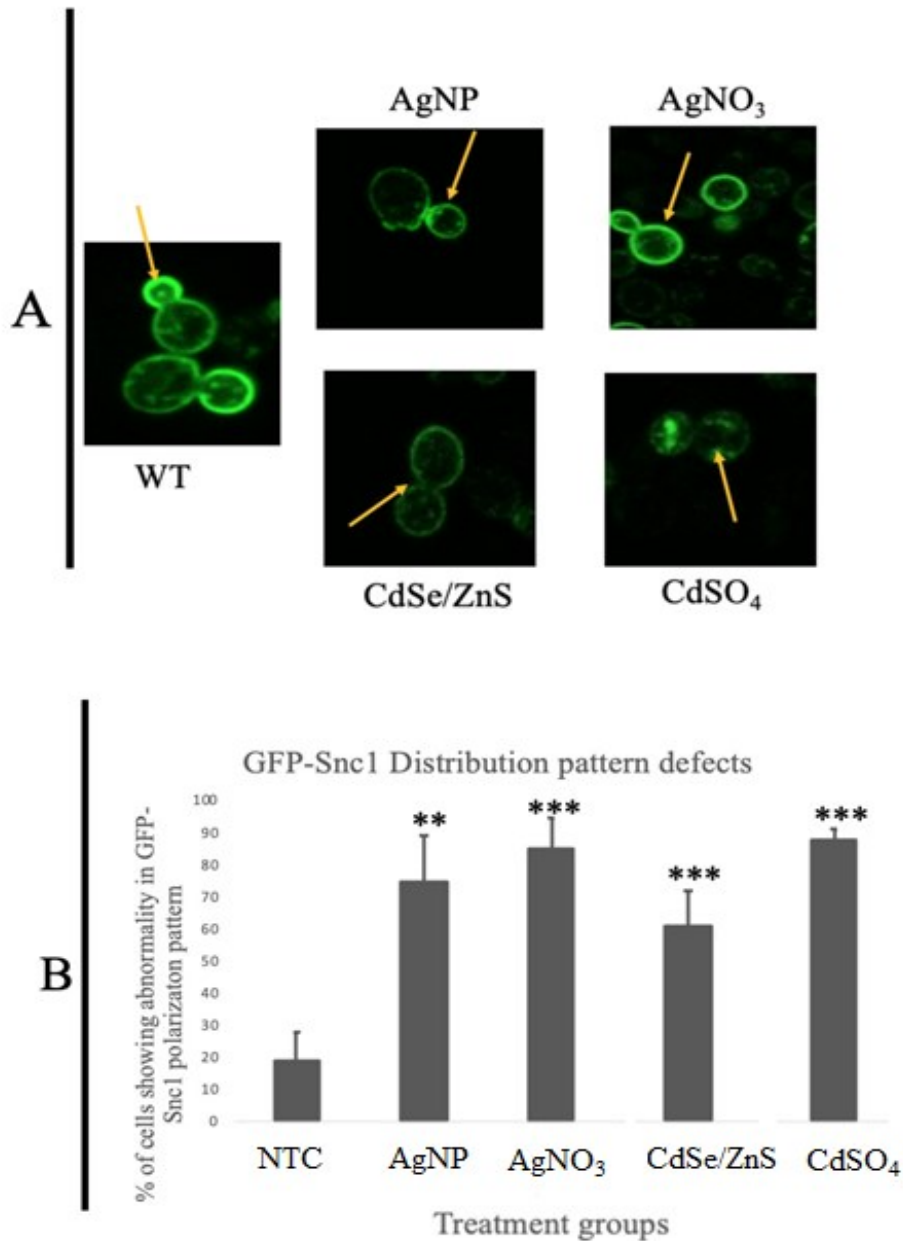


Fig 3: GFP-Snc1 Distribution Pattern Defects. A). Representative images of GFP-Snc1 localization in wild-type and treated WT cells. **B).** Quantification of percentage of abnormal cells in wild type following treatment with 5 $\mu\text{g/mL}$ of AgNP, AgNO₃, CdSe/ZnS and CdSO₄ for 1.5 hr in a shaker at 30°C, 3 individual sets with one sample each set including 30 -50 budding cells with 102 cells of AgNP, 134 cells of CdSe/ZnS, 111 cells of AgNO₃ and 142 cells of CdSO₄ with total number not exceeding 150 cells. *** represents $p < 0.001$ and ** represents $p < 0.01$. WT- Wild Type cells and NTC- Non treated controls.