Roskilde University
International Basic Studies in Natural Sciences

4th semester project
Final Report

Nanoparticle-induced cell death

May, 26th 2011

Group 13: House 13.2
Juliette Tavenier
Evelina Martinenaite
Malgorzata Ewa Dabrowska

Supervisor: Henning F. Bjerregaard
ABSTRACT

Copper (II) oxide nanoparticles (NP) have been shown to have a toxic effect on cells. However, the exact mechanisms of copper oxide (CuO) nanoparticles cytotoxicity are not well investigated. In order to investigate the impact of nanoparticles on the cells, CuO NP (100 nm) were used in vitro. In our experiments, A6 epithelial kidney cells were grown in culture media and exposed to different concentrations of nanoparticles (200μM, 20μM, 2μM) and copper ions (200μM, 20μM, 2μM). NP have been shown to induce oxidative stress. N-acetyl-L-cysteine (NAC) (1mM) was used as an antioxidant pre-treatment prior to exposure to NP.

In order to determine if a short time exposure (1h to 3h) to nanoparticles is sufficient to trigger cell death signaling, two Ringer’s solution with pH 7.3 and pH 5 were used as temporary growth media (for the time of nanoparticle exposure). The actin disrupting agent Cytochalasin D (10 μM and 5 μM) was used in order to inhibit cellular uptake of nanoparticles. To see the effect of NP on the cell cycle FACS analysis was conducted.

Results of the experiments showed no acute cytotoxicity of copper oxide NP on A6 cells and indicated that 7-9 hours are needed for the induction of a significant decrease in cell viability. Cytotoxic effects of copper ions were found significantly lower than that of nanoparticles. We observed a time and dose dependent toxic effect of copper oxide NP on the cells. A delay in cell death induction could be observed when the culture media was supplemented with NAC. Also, the use of a pH5 Ringer’s solution permitted in some cases to remove most of the nanoparticles from the cells’ apical surface and therefore to reduce their toxic effect. Cytochalasin D pre-treatment of the cells seemed to inhibit the uptake mechanisms and postponing cell death. FACS analysis showed a stop in the S phase of the cell cycle upon NP exposure and indicated the cell death mechanism to be apoptosis.
ACKNOWLEDGEMENTS

We would like to express our deep and sincere gratitude to our supervisor, Associate Professor Henning F. Bjerregaard. His ideas and knowledge had a great impact on our work and his constructive comments helped us throughout the project.

We are also deeply grateful to our laboratory technician, Marianne Lauridsen. Her thoughtful support has been of great value during the experimental work.

We warmly thank our opponent group and their supervisors, Associate Professor Annemette Palmqvist and PhD Student Mattia Meli for their detailed review and constructive criticism.
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1. Introduction

Developments in the field of nanotechnology lead to the growing use of nano-sized particles in manufacturing due to their unique characteristics. Nanoparticles are present as parts of packing materials, lubricants, plastics and additives in a variety of skin products and can also be found in significant amounts in industrial exhaust emission. When in contact with materials containing nanoparticles, nano-sized particles may be easily inhaled or ingested. However, nanoparticles are being increasingly introduced without the proper assessment of the risks they impose on the human health. Therefore, there is an ongoing debate on nanoparticles and their effect on the living organisms and the possible adverse health effects. The impact of nanoparticles on living organisms is still the subject of an ongoing research. It has been shown that depending on their chemical nature and size, nanoparticles can be very toxic on the cellular level and even cause cell death (Unfried et al., 2007).

**Aims of the project:**

We hope that our study will help to acquire better understanding of the adverse effects of nanoparticles on living organisms. In order to assess the toxicity of nanoparticles, we investigated the toxic effects of copper oxide nanoparticles on A6 kidney cell line. The cell cycle and the mechanisms of the cell death were taken into account to help identify signaling pathways triggered by nanoparticles. During the experiments we exposed both differentiated and not differentiated A6 cells to nanoparticles and investigated the difference in their responses to assess the effect on differentiated and dividing epithelial tissues in living organisms.

**Problem formulation:** How do copper oxide nanoparticles affect the cell cycle and induce cell death in A6 cells?

Sub-questions:
- What is the toxic effect of the nanoparticles on the cell and how does it depend on the time of exposure?
- What cellular death signaling pathways are triggered by the nanoparticles?
- How do nanoparticles affect cells in different stages of cell life cycle?
2. Background theoretical information

2.1 Copper oxide nanoparticles and copper ions

Nanoparticles are defined as solid particles with a diameter of less than 100nm (Unfried et. al., 2007). Their size allows them to have unique properties, such as unique optical properties, electrical conductivity, magnetic characteristics, hardness, large active surface area, chemical reactivity, and biological activity (Karlsson et al., 2008).

Copper oxide nanoparticles, as metal oxide nanoparticles are used as industrial catalysts and are also presently often used in commercial products, such as antimicrobial preparations, semiconductors, heat transfer fluids or intrauterine contraceptive devices (Fahmy, Cormier, 2009). However, CuO NP have been shown to have a specific toxic effect on living organisms: induction of hepatotoxicity and nephrotoxicity has been shown in rats and toxic effect on epithelial cell line cultures (eg. human laryngeal epithelial cells) (Fahmy, Cormier, 2009).

As mentioned by Unfried et al. (2007), it is possible that metal ions are released from impurities on the nanoparticles’ surface. The dissolution of copper ions (Cu$^{2+}$) from the copper oxide nanoparticles would be even more damaging to the cells, as copper ions are known to have harmful effects. Indeed, studies have shown that even though copper is an essential element for life, it is toxic at high concentrations. Copper ions are responsible for increased reactive oxygen species levels, DNA damage, and induction of apoptosis (Rana, 2008).

2.2 Cellular uptake mechanisms of nanoparticles

The different uptake mechanisms

Although the cellular uptake mechanisms of nanoparticles are yet to be fully investigated, many studies have been conducted, and it already clearly appears that the uptake mechanisms are specific to each size, surface charge, chemical composition and shape of nanoparticles, as well as to each type of cells exposed to the nanoparticles. It can also be
possible that more than one mechanism is responsible for nanoparticles uptake (Unfried et al., 2007). Among those several pathways for the uptake of nanoparticles which have been investigated, we find: endocytosis, diffusion through the membrane, phagocytosis and macropinocytosis (Unfried et al., 2007).

Figure 1: Mechanisms for the uptake of nanoparticles by the cells: (1) phagocytosis, (2) macropinocytosis, (3) clathrin-mediated endocytosis, (4) non-clathrin, non-caveolae mediated endocytosis, (5) caveolae-mediated endocytosis, (6) diffusion. (Unfried et al., 2007).

In figure 1, different mechanisms for the uptake of NP by the cells are described. The numbers between brackets in the following text, refer to the numbers and letters in the figure.

Phagocytosis (1) is the process during which materials larger than 250nm are ingested by the cell. The material is gradually surrounded by a part of the plasma membrane of the cell, which invaginates before pinching off to form a vesicle. In the case of phagocytosis (1), the formed vesicle is called a phagosome (Alberts et al., 2007). This phagosome goes through an early (1A) and a late (1B) stage before turning into a lysosome which will break down the ingested material (1C) (Unfried et al., 2007).

The second described mechanism is the macropinocytosis (2). It is actually a pinocytosis mechanism applied to the ingestion of larger amounts of fluid or larger particles than in a regular pinocytosis. The particles are engulfed into vesicles: the macropinosomes (2A); which, just like the phagosomes, undergo several maturation steps before formation of a lysosome (1C) (Unfried et al., 2007).

Endocytosis is a complex mechanism which can occur through several pathways: clathrin- (3) and non clathrin-mediated (4) or caveolae-mediated endocytosis (5) (Unfried et al., 2007). The clathrin-mediated (3) pathway is also known as clathrin-dependent
endocytosis, because the cellular uptake of particles takes place in clathrin-coated pits (3A); clathrin-mediated endocytosis (CME) requires other specific proteins such as AP-2 and dynamin. The role of clathrin is to stabilize the membrane structure during the budding, and the AP-2 protein associated to other proteins is thought to be facilitating the development of the curvature. Although the exact mechanism responsible for the bending of the membrane is still unclear, certain proteins such as the BAR and F-BAR proteins seem to have the ability to bend a flat membrane surface (Lundmark and Carlsson, 2010). Before clathrin-coated vesicles are formed (3B), the neck connecting the vesicle to the membrane will become very thin, allowing rings of dynamin to assemble around the neck, and by constriction, cleave off the formed vesicle inside the cell. When the vesicle pinches off, the two separated parts of the membrane are brought together and fuse, sealing the membrane, see figure 2, (Alberts et al., 2007). Once the vesicle is fully formed and detached from the membrane, it will be uncoated from its clathrin (3C).

![Figure 2: Role of dynamin in pinching off clathrin-coated vesicles in CME. Dynamin helixes assemble around the neck, pinch off the vesicle from the membrane and seal the two separated parts of the membrane (modified from Alberts et al., 2007).]

Then the particles will be processed by early endosomes (3D), multivesicular bodies (3E) and late endosomes (3F) before eventually reaching the lysosome stage (1C) where the particle will be degraded (Unfried et al., 2007).

The non-clathrin mediated endocytosis is also called clathrin-independent endocytosis (4); very little is known about the pathways resulting in this specific type of endocytosis. Studies have shown that the clustering of lipids from the cell’s membrane is necessary for this type of endocytosis. The rafts formed by the clustering are made of cholesterol, sphingolipids and glycosphingolipids; all three of these raft components are strongly chiral, and therefore, the mirror images of these molecules have a different chemical structure even though their composition remains the same. The presence of these chiral molecules in the cell’s membrane allows it to alter its shape; this associated with tilts or inclines in the lipid chain or head groups orientation of the molecules (due to specific constitution and physical characteristics of the rafts) can result in the formation of buds or tubules in the membrane (Sarasij et al.,
The association of additional proteins (such as dynamin, or BAR) to this curvature could possibly lead to the formation of vesicular (4A) or tubular structures as well as their scission from the membrane (Doherty and McMahon, 2009).

Similarly to the non-clathrin non-caveolae mediated endocytosis, the caveolae-mediated endocytosis (5) occurs as a result of the formation of rafts (5A) of the membrane lipids, such as cholesterol or glycosphingolipids, which then invaginate forming pear-shaped vesicles when they are associated with the caveolin protein (Cavalli et al., 2001). These vesicles, the caveosomes (5B), are also pinched off from the membrane with the help of dynamin (Alberts et al., 2007) and can eventually transfer the ingested particles in the endoplasmic reticulum (5C) or in the cytosol (Unfried et al., 2007).

Finally, it is possible that nanoparticles enter the cells simply by diffusion through the membrane (6), (Unfried et al., 2007).

**The role of actin in endocytosis**

All of the previously mentioned uptake mechanisms (except for the diffusion) involve actin. Actin is one of the major components of the cytoskeleton, and it plays an important role in the endocytotic mechanisms. Even though its precise function remains unclear, actin is believed to participate in the cells’ spreading during the invagination of the particle (Cavalli et al., 2001).

The actin protein is able to polymerize into helical actin filaments, also known as actin microfilaments (MF). Actin dimers (two molecules) and trimers (three molecules) are unstable, unlike the polymers. The actin filaments are polar; therefore, their ends are distinct: one end (the barbed end) grows faster than the other: pointed end (Pollard and Cooper, 2009), see figure 3.

*Figure 3: Structure and polymerization of actin. A: the actin molecule. B: polymerization of an actin filament. Growth rate is higher at the B (Barbed) end than at the P (pointed) end. (Modified from Pollard and Cooper, 2009)*
As mentioned earlier the precise role of actin in endocytotic processes is still unclear; there exist several models aimed to explain its role in endocytosis, two of them are presented in figure 4. One theory states that some proteins involved in endocytosis also regulate the assembly of actin in the endocytotic sites. For instance, in the CME process, before the vesicle is pinched off, actin is believed to interact with clathrin and help the vesicle move away from the membrane, see figure 4. Experiments also demonstrated that actin contributes to the invagination of the pits and to the shaping of the vesicles (Galletta et al., 2010).

![Figure 4: Models for the assembly and function of actin in clathrin-mediated endocytosis (CME). In both (a) and (b) the membrane curvature is initiated by clathrin, and by the F-BAR containing protein Syp1. Invagination proceeds with the help of other BAR containing proteins, such as Bzz1. The activation of Arp2/3 complexes initiates the creation of actin filaments and branches on these filaments. In the first model (a), the actin MFs are attached to the membrane, permitting other endocytotic proteins to bind to this actin network and invaginate further into the cell. Then the proteins Rvs161 and Rvs167 induce the pinching off of the vesicle. In the second model (b), the actin MF are created at the sides of the forming vesicle, growing ends pushing on the membrane of the vesicle, elongating it and thus helping the Rvs161/Rvs167 proteins in their scission work. Finally, the actin MFs can drive movement of the vesicles away from the membrane. This second models is a better fit to the process occurring during CME in mammalian cells. (Galletta et al., 2010)](image)

**Cytochalasin D as an endocytosis inhibitor**

We stated earlier that actin is playing a major role in the endocytosis process; therefore, a way to inhibit endocytosis could be to disrupt the polymerization of the actin microfilaments.

Cytochalasin D (CD) (figure 5) is derived from a group of fungus metabolites; it can easily penetrate the cell’s membrane and cause it to become less stiff and to round up. CD is
known to bind to the barbed end of actin filaments; therefore, inhibiting its growth at this end. The stoichiometry of the binding is of about one cytochalasin per actin filament. Some experiments also show that CD can shorten the actin MF; this would be done by a cytochalasin binding to an actin subunit in the interior of the filament, breaking it in two parts, this process is called severing. Cytochalasin can also bind to actin monomers and dimers (Cooper, 1987).

It has been shown that in cells exposed to CD, during the CME process, the clathrin-coated pits are unable of pinching off from the membrane; and therefore, an abnormally high number of these clathrin-coated vesicles attached to the membrane are observed (Apodaca, 2001).

Cytochalasin D does not only disrupt the inner cellular structures involving actin, but also affects the cell surface. Figure 6 shows the effect of cytochalasin D on actin microridges present at the surface of the epithelial A6 kidney cells. After they are exposed to the chemical, the cells are lacking the microridges – they are disrupted by cytochalasin D since actin is involved in their formation. And because the cilia are made of microtubules, and not of actin, they are still present after exposure (Schropfer et al., 2000).

![Figure 5: Chemical structure of Cytochalasin D](sigmaaldrich.com)

![Figure 6: Microridges on the surface of A6 epithelia kidney cells. 1) Untreated confluent monolayer of cells, microridges and cilia (red arrowhead) are visible. 2) Confluent monolayer of cells treated with Cytochalasin D; some cells appear without microridges, but cilia (arrowhead) are still presents. (Schropfer et al., 2000)
2.3 Cell cycle

The cell cycle is the sequence of occurrences taking place in the cell leading to its growth and culminating in its replication by duplication. The cycle is divided into two major phases: interphase and mitosis. Mitosis (M) is the phase during which the cell replicates. For the duration of interphase, which is the longer of the two phases, the cell does not divide. The interphase is further divided into 3 sub-phases: G$_1$ (first gap), S (synthesis) and G$_2$ (second gap), see figure 7.

The role of the G$_1$ phase is to allow the cell to grow before replication of the chromosomes (Alberts et al., 2007). At this stage, the cells carry out all of the biosynthetic activities that were slowed down during the M phase (e.g. obtaining of nutrients, growth, and synthesis new organelles). Throughout this stage the cell requires high amounts of enzymes and structural proteins, therefore large amounts of proteins are synthesized and cellular metabolic rate is elevated. Cell growth may be inhibited by a lack in nutrient supply, unsuitable temperatures, and other inhibiting factors (Meraldi and Nigg, 2002). G$_1$ starts just after the end of the replication and lasts until the preparation for the DNA synthesis is arranged. Then, the checking mechanism is performed.

During the next step of the cell cycle, the DNA synthesis phase (S), the replication of the chromosome by semiconservative duplication takes place (Figure 8). The checking point is very important in this stage of the cell cycle as a precise and accurate prevention of the
genetic mutations and abnormalities is necessary for the cell’s survival. When the checking mechanisms detect any harmful abnormalities in the copied DNA, the cell may enter the special rest cycle (G₀) or enter apoptosis. The majority of the cells actually spend most of their life in the G₀ state, but they can be “called back” from the G₀ state, for instance in case of injury (Campbell et al., 2008). At the end of the chromosome replication process each chromosome consist of two sister chromatids and the cell’s genetic information (DNA) has been doubled (Meraldi and Nigg, 2002).

A successful completion of the S phase allows the cell to enter the third and last stage of interphase, the second gap (G₂) which lasts until the cell proceeds to the mitotic phase. It is important for the cell to accumulate nutrients since it undergoes a rapid growth, an enhanced protein synthesis rate, and prepares for the cell division. Moreover during the G₂ phase, the centrosomes come to maturation and microtubules are produced – both further involved in mitosis. Some species (predominantly young Xenopus embryos) skip the G2 phase and continue straight into the mitosis (Alberts et al., 2007).

Mitosis (the M phase) is the essential mechanism for cell division. Although the cells spend the majority of their lives in the interphase (Figure 7), mitosis is fundamental since it is during this phase that the cell is passing on its genetic material (DNA) to the new generation of cells. It is also during this phase, that chromosomes are segregated and cell division involving the duplication of nuclei and cytoplasmic division called cytokinesis take place. Duplication is the only way of cell multiplication. Stopping cell division will cause cell death (Alberts et al., 2007).

Mitosis is highly controlled by the regulatory proteins and is divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase. During the prophase, the chromatids of each chromosome pair up with their sister chromatids, and the centrosomes, with the help of the microtubules, move away from each other. The prometaphase is
identifiable by the fragmentation of the nuclear envelope, allowing the chromosomes to flow out of the nuclei and into the cytosol. During metaphase, the centrosomes reach the opposite ends of the cells and the chromosomes align on the metaphase plate (the imaginary plate located in the center of the cell, at equal distance from each centrosome). Following comes the anaphase, during which the two sister chromatids of each chromosome come apart and start moving towards opposite end of the cell, which starts elongating. Finally comes the telophase, when the two daughter cells and their nuclei start forming (Campbell et al., 2008).

After mitotic division, the newly formed cells can proceed to the $G_1$ phase. Cells may also exit the cell cycle by going into the $G_0$ phase which is the rest phase. If cells do not return into the cycle, nor go into $G_0$ state, they consequently perform apoptosis (Alberts et al., 2007).

To perform the checking mechanism, the cell cycle is governed by the system of regulatory proteins. One of the cell cycle regulation mechanisms involves a certain type of proteins called cyclin-dependent kinases (Cdks). Activity of cdks rises and falls through the progression of cell cycle. An increase in enzymatic activity of cdks results in phosphorylation of cellular proteins that catalyze major events in cell cycle. As their name indicates, the activity of cdks relays on the presence of cyclin proteins. Cyclins are divided into four classes: $G_1/S$, $S$, $M$ and $G_1$. Each class of cyclins has a specific function and ensures regulation at specific phases in the cell cycle (Figure 9). $G_1/S$ cyclins bind to cdks at late $G_1$ phase and ensure the transition through $G_1$ checkpoint. $S$ cyclins activate cdks after the $G_1$ checkpoint and retain their activity until the mitosis, as well as contribute to early mitotic events. $M$ cyclins bind cdks at $G_2/M$ checkpoint and stimulate the beginning of mitosis. $G_1$ cyclins control the activity of $G_1/S$ cyclins (Alberts et al. 2007).

**Figure 9:** Level of cyclins during the progression of cell cycle. (Alberts et al. 2007)
2.4 Oxidative stress

Oxidative stress is considered to be one of the most harmful cellular effects induced by exposure to nanoparticles; it is the cause of significant DNA damages, as well as lipid and protein oxidation (Martindale and Holbrook, 2002). The oxidative damages occur when there is an imbalance between oxidants and antioxidants: when the levels of reactive oxygen species (ROS) in the cell are too high to be neutralized successfully by the cells’ natural antioxidants (Zafarullah et al., 2003). The cellular response to the presence of reactive oxygen species is very variable according to the cell type, duration of exposure and level of the ROS inside the cell (Martindale and Holbrook, 2002). The response ranges from an increased cell proliferation for relatively low doses of ROS, to growth arrest and ageing at intermediate doses, and to cell death for high levels of ROS (Figure 10).

**Reactive oxygen species in a healthy cell**

Reactive oxygen species are continuously generated in the mitochondria during oxidative phosphorylation. Oxidative phosphorylation is the process of degradation of fats, carbohydrates and amino acids occurring in the mitochondria, and which is driving the synthesis of ATP (Nelson and Cox, 2008). Reactive oxygen species are highly reactive free radicals, such as superoxide (O$_2^-$), hydroxyl free radical (‘OH), or hydrogen peroxide (H$_2$O$_2$). Their high reactivity is due to their unpaired electron (Figure 11).

![Diagram of cellular response to different reactive oxygen species levels](image)

**Figure 10:** Cellular response to different reactive oxygen species levels. (Martindale and Holbrook, 2002).

![Diagram of reactive oxygen species](image)

**Figure 11:** Reactive oxygen species: Oxygen/Superoxide, Hydroxyl ion/Hydroxyl free radical, (Hydrogen) Peroxide (Bowen R, 2003).
Radicals are generated by simple oxidation and reduction processes (Unfried et al., 2007), such as the reduction of oxygen in the mitochondria (Equation 1):

\[ O_2 + e^- \rightarrow O_2^- \]  (1)

The \( O_2^- \) free radical formed by this reaction is highly reactive and leads to the formation of an even more reactive hydroxyl radical \( \cdot OH \). However, cells have natural defense mechanisms to protect themselves from the harmful effects of reactive oxygen species. Some specific enzymes are endogenous (such as catalase, superoxide dismutase, vitamin C and E, glutathione peroxidase); they are produced by the cells and inhibit the oxidation mechanisms producing ROS. Those enzymes are called anti-oxidants: they protect the cells by interacting with the free radicals, and inhibiting oxidation reactions.

In order to defend the cells from oxidative damage due to the superoxide free radical, the enzyme superoxide dismutase catalyzes the reaction yielding hydrogen peroxide, and \( O_2 \) (Nelson and Cox, 2008) (Equation 2):

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]  (2)

The hydrogen peroxide formed by this reaction is reduced to water by glutathione peroxidase (which is derived from glutathione (GSH)) to avoid any harmful effect the oxidative damage by the superoxide free radical could have on the cells (Nelson and Cox, 2008) (Equation 3):

\[ H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \]  (3)

The oxidized glutathione (GSSG) is then reduced to its initial form (GSH) by the glutathione reductase, which uses electrons from NADPH (oxidizing it to NADP\(^+\)) generated in the mitochondria (Nelson and Cox, 2008). In addition, the catalase enzyme can reduce \( H_2O_2 \) to \( O_2 \) and \( H_2O \) very rapidly (Kelly et al., 1998).

However, the hydrogen peroxide may also react with superoxide, creating the hydroxyl radical \( \cdot OH \), this reaction is called the Haber-Weiss equation (4) (Kelly et al., 2008):

\[ O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2 \]  (4)

There also exist some exogenous (coming from the outside) compounds that can play the role of antioxidants, such as N-acetyl-L-cysteine (NAC). N-acetyl –L-cysteine contains a
thiol group (-SH) (figure 12), this group gives it the ability to reduce free radicals; NAC is also a precursor for glutathione (Zafarullah et al., 2003).

![Chemical structure of NAC](image)

**Figure 12:** Chemical structure of NAC (Sigma-Aldrich, 2011)

**Reactive oxygen species in a cell exposed to nanoparticles**

Dissolution of metal ions, from the nanoparticles, in their reduced form (Me^{2+}; or in the case of copper (II) oxide: Cu^{2+}), and physical interaction with specific cellular structures involved in the cell’s defense mechanism against ROS, could explain the induction of excessive levels of ROS by nanoparticles (Unfried et al., 2007).

In the presence of high levels of metal ions (such as copper) in their reduced form, the efficient reduction of H_{2}O_{2} by peroxidases (such as glutathione peroxidase in equation 3) will be disrupted, and the Fenton-reaction (Equation 7) is likely to occur, producing the very reactive hydroxyl radical ‘OH (Moriwaki et al., 2008)(Equation 5-7):

\[
H_{2}O_{2} + Cu(II) \rightarrow CuOOH^+ + H^+ \quad (5)
\]

\[
CuOOH^+ \rightarrow Cu(I) + O_2^- + H^+ \quad (6)
\]

\[
Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + \cdot OH \quad (7)
\]

The presence of metal ions can also trigger the production of ‘OH free radical, as they act as catalysts for the Haber Weiss reaction (in equation 4). The Haber-Weiss reaction catalyzed by copper is presented in equation 8 and 9, where equation 9 is the Fenton reaction (Kelly et al., 1998):

\[
O_2^- + Cu(II) \rightarrow O_2 + Cu(I) \quad (8)
\]

\[
Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + \cdot OH \quad (9)
\]
The defense mechanisms of the cells against those reactive species are summarized in figure 13.

![Diagram of ROS production, defense mechanisms and effects of free radicals on the cell.]

**Figure 13**: ROS production, defense mechanisms and effects of the free radicals on the cell. GSH: glutathione, GSSG: glutathione disulfide (oxidized form of GSH) (Modified from Kelly et al., 1998).

Even though it is still not clear how nanoparticles are interacting at the cellular level, it is thought that they are contributing to ROS generation inside the cells. Some structures, such as the endoplasmic reticulum, specific enzyme complexes on the cell membrane (in particular the NADPH oxidases), and mitochondria, are considered to be principal targets of nanoparticle-induced reactive oxygen species. These interactions between NP and subcellular structures result in the generation of reactive oxygen species along with an increase in calcium ions $\text{Ca}^{2+}$ released by the mitochondria and endoplasmic reticulum (Unfried et al., 2007) (figure 14).

The increase in $\text{Ca}^{2+}$ levels in the cells may trigger the activation of specific enzymes, such as endothelial (or neuronal) nitrogen monoxide synthases. The stimulation of such enzymes results in the formation of nitrogen monoxide ($\text{NO}^-$) which can react with superoxide and generate peroxynitrite (Unfried et al., 2007) (Equation 10).

$$\text{NO}^- + \text{O}_2^- \rightarrow \text{ONOO}^- \quad (10)$$

The exposure of cells to nanoparticles results in an increase in the generation of both reactive oxygen species and reactive nitrogen species; which at too high doses can be lethal to
the cells (Figure 10). Indeed, an excessively important level of ROS will require more antioxidants than the cell can produce in order to defend itself and this will cause oxidative damage to the cell and its content (Unfried et al., 2007).

![Figure 14](image)

**Figure 14:** Physical interactions of nanoparticles with subcellular compartments as a determinant of the type of reactive oxygen species generated. a) nanoparticle interaction with NAPDH oxidase (NOX) complexes generating superoxide. b) interaction with mitochondria and generation of superoxide and calcium ions. c) interaction with endoplasmic reticulum and generation of calcium ions. The generation of $\text{Ca}^{2+}$ ions results in the formation of nitrogen monoxide (NO) which can react with superoxide and form peroxynitrite (ONOO$^-$). (Unfried et al., 2007).

**Oxidative stress and damage to the cells**

The oxidative stress generated by nanoparticle-induced ROS level increase affects both the mitochondrial and nuclear genomes. It has been established that the highly reactive oxygen species, such as the hydroxyl radical ‘OH, attack the mitochondrial genome. And as it is the mitochondrial DNA (mtDNA) which is encoding the majority of the proteins playing an important role in the electron-transport chain, along with two subunits of the ATP synthase, the enhanced ROS generation associated to GSH depletion may disrupt the electron-transport chain, as well as lead to mitochondrial DNA mutagenesis (Unfried et al., 2007). Furthermore, because the mtDNA is located close to the electron transport chain, and because the electron transport chain is where most of the free radicals originate from, the mitochondria are very vulnerable to oxidative damage (Orrenius et al., 2007). Moreover, it has been shown that those impairments are related to an increase in ROS generation; thus establishing a vicious cycle leading to the loss of mitochondrial function, and in the long run, to apoptosis (Unfried et al., 2007).
The nuclear genome is also affected by the oxidative stress in several ways: initiation of mutations, induction of DNA cell cycle arrest, and apoptosis. The DNA damage and mutations are due to an oxidative attack similar to that affecting the mtDNA and can for example result in the breakage of the DNA strands. However, the cells are equipped with repair mechanisms; they detect any impairment and stimulate a system of signaling pathways as a response to the damage (the DNA damage response). These structured response mechanisms starts at the cell cycle check-point, and trigger a DNA repair action or induce programmed cell death (apoptosis, which is very likely to happen in the case of double DNA strand break). Nucleotides can be oxidized by the free radicals such as the peroxide ion (Figure 15), and their oxidized form is an important cause of mutations when it assimilates in the DNA strands. The repair mechanism associated with this kind of damage is called base excision repair. There exist other repair pathways, such as nucleotide excision repair and mismatched repair (Barzilai and Yamamoto, 2004). They act by removing the defective part of the DNA and replacing it by a “healthy” one. As mentioned earlier, when cells detect damage, and in particular DNA damage, they can go into a permanent $G_0$ state, in order to have more time to be repaired or they can undergo apoptosis (Barzilai and Yamamoto, 2004). Another repair mechanism used against ROS induced DNA damage involves the activation of the protein 53 (also called p53). This p53 protein is known to initiate apoptosis (Martindale and Holbrook, 2002).

Lipids, and particularly polyunsaturated (more than one double bond in the fatty acid chain) fatty acids in phospholipids of both the cell’s and mitochondria’s membrane, are also targets of oxidative stress. They undergo an oxidation process, usually referred to as lipid peroxidation (Orrenius et al., 2007). This oxidation of the lipids is induced by the OH hydroxyl radical which takes electrons from the lipid in order to form a water (H$_2$O) molecule. This then causes a chain reaction during which the lipid will be further degraded into lipid peroxide see figure 15 (Clark, 2008). The presence of lipid peroxides affects the mitochondrial metabolism in many ways: respiration and oxidative phosphorylation, along with the Ca$^{2+}$ buffering capacity and membrane properties of the mitochondria can be altered (Orrenius et al., 2007).

Proteins as well are subjected to the adverse effects of ROS. They also go through an oxidation reaction (figure 15) (Clark, 2008). Moreover the free radicals can bind to amino acids such as arginine, lysine, threonine and proline, resulting in the formation of protein carbonyls, alcohols, and peroxides, which have the ability to perturb the tertiary structure of
proteins leading to unfolded or misfolded proteins. These non-functional proteins are highly hydrophobic and therefore have a tendency to form protein-protein interactions which is damaging to the cells (Orrenius et al., 2007).

Figure 15: Damaging effects of reactive oxygen species on: a) lipids, b) proteins, c) DNA (modified from Clark, 2008)

**Oxidative stress and cell death signaling**

There exist several cell death pathways, leading to different cell death mechanisms, among them are apoptosis and necrosis. We will focus on the relation between abnormally high levels of reactive oxygen species and these two specific cell death mechanisms. They differ in many aspects, such as the morphological aspect and the signaling.

Apoptosis, or programmed cell death, is the process during which cellular agents degrade the DNA, organelles and other cytoplasmic components, killing the cell, in order to protect neighboring cells. Apoptosis is identifiable by its morphological appearance: formation of vesicles, loss of the electrochemical potential of the mitochondrial membrane, darkening and fragmentation of the genetic material in fragments of distinct sizes. Another specific characteristic of apoptosis is the decrease in cell volume. Shrinkage of the cells is occurring during the beginning of the apoptotic process, and is due to the loss of ions (K\(^+\), Cl\(^-\), Na\(^+\)) from the cytosol and an associated loss of water. The ion transport through the
membrane channels causes the cells to lose about 30% of its water content. It is thought that the shrinkage of the cells is a trigger to apoptosis, since it usually occurs prior to the activation of caspases and cytochrome c release which are enzymes and proteins responsible for apoptosis (Lambert et al., 2008).

There exist many different pathways leading to apoptosis, which involve around 15 different specific enzymes called caspases (Campbell et al., 2008). The mitochondria play a major role in apoptosis initiation. When they are under excessive stress, such as oxidative stress due to ROS levels, their membranes become more permeable in order to allow cytochrome c to escape. Cytochrome c is a protein found in the inner membrane of the mitochondria, it plays an important role in the mitochondrial electron-transport chain. However, when it is released in the cytosol (due to an increased permeability of the mitochondria’s membrane under stressed conditions), it has a completely different role (Alberts et al., 2007). It will form a complex with a protein called apoptotic protease-activating factor-1 (Apaf-1). This complex then forms an apoptosome (Figure 16). The Apaf-1 in this apoptosome will convert inactive procaspase-9 into active caspase-9. The activation of the caspase-9 initiates a cascade of other activations: one caspase activating a second one, activating in its

Figure 16: Role of cytochrome c in apoptosis: Cytochrome c release in the cytosol, formation of apoptosome and activation of caspase-9, -7 and -3, leading to apoptosis of the cell. (Nelson and Cox, 2008)
turn a third one and so forth. Here the caspase-9 will activate the caspase-3 and caspase-7, which both will induce cell death (Nelson and Cox, 2008).

Reactive oxygen species can also trigger apoptosis by the activation of the p53 protein. Excessive DNA damage due to oxidative stress predominantly contributes to the activation of p53, which it is known to result in either apoptosis or senescence of the cell. The pathways leading to p53 up-regulation are numerous and complex. Besides, even though many apoptosis-related proteins have been identified as targets of the p53 protein, there is still a lot to investigate about the precise apoptosis induction mechanism. Though, it its known that the activation of p53 leads to additional production of ROS as well as the down-regulation of anti-apoptotic and activation of pro-apoptotic proteins, which then results in the induction of apoptosis (Martindale and Holbrook, 2002).

Another cause of apoptosis related to nanoparticles toxicity involves increased calcium ion (Ca$^{2+}$) levels. Indeed, it has been stated earlier that interaction between NP and specific cellular structures results in the generation of reactive oxygen species along with an increase in Ca$^{2+}$ released by both the mitochondria and endoplasmic reticulum (see Reactive oxygen species in a cell exposed to nanoparticles). When Ca$^{2+}$ is released into the cytosol, some specific proteases are activated, leading to apoptosis (Zong and Thompson, 2006).

It has also been reported that the release of cytochrome c in the cytosol could result in necrosis of the cell (Unfried et al., 2007). However, necrosis is not a programmed process. We mentioned earlier that ROS affect the mtDNA, and therefore the ATP synthesis (see Oxidative stress and damage to the cells), resulting in a reduction of ATP levels in the cell. This depletion in ATP can be at the origin of the opening of channels in the cytoplasmic membrane, which is under normal conditions selectively permeable to anions. Such an alteration of the membrane permeability will allow cations to flow inside the cell, causing osmotic stress and resulting in the cell’s swelling, and eventually in its rupture. Moreover, the integrity of cell’s membrane can be disrupted due to lipid peroxidation by NP, leading to an influx of Ca$^{2+}$ in the cytosol, and therefore to necrosis (Zong and Thompson, 2006). The over-expression of specific proteins such as PARP (Poly (ADP-ribose) polymerase) is a response from the cells to the damage induced to their genetic material by NP. PARP acts by reducing the NAD$^+$ (nicotinamide adenine dinucleotide) levels in the cells; thus forcing the cell to combine nicotinamide and two ATP molecules in order to synthesize NAD$^+$ and to reestablish
the NAD$^+$ levels. This leads to a depletion of ATP in the cells, further leading to necrosis (van Wijk and Hageman, 2005).

Figure 17 summarizes the damages induced by the nanoparticles on the cell’s nucleus and mitochondria and on the genetic material they contain.

![Figure 17: Nanoparticle-induced oxidative stress and its effect on the mitochondria and nucleus](image)

(Unfried et al., 2007).

2.5 Cell culture

The A6 cell line is an epithelial cell line from a distal part of a nephron (see figure 18). Originally, the cell line is derived from African clawed frog *Xenopus laevis*, but it has been shown to display structural and functional properties of mammalian distal epithelium cells (Bjerregaard HF, 2006).

The A6 cell line exhibits contact inhibition of cell division, since further cell
division stops after a confluent monolayer is formed. This cell line has a doubling time of around 20 hours. Dividing A6 cells usually have a fibroblast-like shape with characteristic membrane extensions. Another clearly visible characteristic of proliferating A6 cells is a presence of 2 or more light dots (see figure 19). These dots are not observed in confluent cell layer.

In a confluent A6 cell monolayer, cells form tight junctions. The cellular membrane facing the culture media is referred to as the apical membrane. The membrane turned towards the bottom of the plastic culture flask is referred to as basal membrane.

Differentiated A6 cells transport Na\(^+\) and Cl\(^-\) ions across the membrane through ion channels. Active transport of Na\(^+\) ions across the epithelium monolayer and subsequent diffusion of Cl\(^-\) ions results in water inflow into the area between the basal membrane and the culture flask. This process leads to an elevation of the cell layer and to the formation of domes which can be clearly seen through a light microscope (see figure 20).
Another typical structure formed by A6 cells in a confluent monolayer is the elevated areas, which have a tube-like structure. These structures are referred to as “balloons” (see figure 21).

As many other epithelial cells, the A6 cells are covered with a cell coat, or glycocalyx (Figure 22), consisting of carbohydrates (mainly oligosaccharides) bound to the lipids and
proteins of the cells’ membrane. Sialic acid can be added to the oligosaccharide chains, giving them a negative charge. The role of this cell coat is to protect the cells against physical and chemical injuries, as well as enabling the cells avoid any undesirable protein-protein interactions (Alberts et al., 2007).

Figure 22: Electron image of the apical surface of the epithelial cell (Karp 2010)
3. Materials and Methods

3.1 Chemicals

The 100-nm copper oxide (CuO) nanoparticles used in our experiments were suspended in water, stock solution concentration – 0.0177M. Other stock solution concentrations: CuCl₂ (in water) – 0.019M; cytochalasin D from Sigma-Aldrich (see appendix 5) (in DMSO) – 2mM; NAC from Sigma-Aldrich (see appendix 5) (in Ringer’s solution) – 50mM. Cell culture media composition: 25% water, 10% fetal calf serum, 63% Dulbecco’s Modified Eagle’s Medium (DMEM), (see Table 1 in appendix 1), 2% penicillin/streptomycin.

3.2 Cell culture treatment

A6 frog kidney cells were used for this study to observe cell response when exposed to copper oxide nanoparticles and copper ions (CuCl₂).

The A6 cell line was cultured in 25 cm$^3$ flasks at a density of up to 5x10$^5$ cells per mL (producing 1x10$^6$ cells/mL cell suspension during subcultivation), in 10mL cell culture media. The cells were incubated at 26°C with 5% CO$_2$. The media was changed every 3-4 days and the cells were subcultivated around every 7 days, when they formed a confluent monolayer and dome formation was observed (sign of a complete differentiation of the cell line).

Subcultivation

After the culture media was replaced with 2mL of a trypsin solution (containing trypsin, EDTA, EGTA), the cells were incubated for 3 to 5 minutes at 37°C with 5% CO$_2$ until they started loosening (observable through a microscope). Then, 1.7mL of the trypsin was removed (leaving 300µL) and the cells were placed in an incubator at 37°C for 2 to 5 minutes. The flask was gently “shaken” in order to obtain single cells. 5mL of media at room temperature was added in order to obtain solution with a density of around 1x10$^6$ cells/mL. 1mL of this cell solution (with further addition of 9mL of growth media) was used per new cell flask produced. A solution of the same concentration (1x10$^6$ cells/mL) was also used to fill in a 24-well plate: 20µL or 30µL of cell suspension per well with addition of 1mL of culture media.
Experiments were conducted in 24 well plates, where the cells were cultured in 1mL culture media at a starting density of $2 \times 10^4$ or $3 \times 10^4$ cells/mL ($20 \mu$L or $30 \mu$L of $1 \times 10^6$ cells/mL). Prior to the experiments, the cells grown in 24 well plates were graded for confluence on the scale from 1+ to 3+ (see figure 23).

**Figure 23**: A6 cell culture confluence, right to left: 1+, 2+, 3+.

### 3.3 Experiment design

#### 3.3.1 Copper oxide nanoparticles and copper ions toxicity on a non-differentiated confluent cell layer

The purpose of the experiment was to compare the toxic effect of CuO nanoparticles and copper ions ($\text{Cu}^{2+}$). 200µM, 20µM and 2µM CuO NP and CuCl$_2$ solutions in culture media were prepared.

The experiment was conducted on cells grown for 4 days with 1mL of media in a 24-well plate and forming a confluent layer at the bottom of the plate. For control, the media was carefully removed, and replaced by fresh media. In 3 of the wells, the media was replaced by 1mL of the 200µM CuO NP solution, in 3 other wells, the media was replaced by 1mL of the 20µM CuO NP solution, and in 3 other wells, 1mL of the 2µM CuO NP solution replaced the media. The same process was conducted with the CuCl$_2$ solutions on the 9 remaining wells (3 wells with 1mL 200µM, 3 with 1mL 20µM, 3 with 1mL 2µM of the CuCl$_2$ solution) (figure 1 in appendix 3). Pictures of the cells were then taken after 3h, 6h, 24h, 48h, 72h, 96h and 168h with the LEICA DM IRB inverted light microscope.
3.3.2 Acute toxicity of copper oxide nanoparticles on non-differentiated single cells

The experiment was conducted on single cells with a starting density of $3 \times 10^4$ cells/mL, which had been cultured for one day in a 24 well plate. The media was carefully removed from 3 wells, and replaced with 1mL of a 200µM CuO NP in media solution, the 3 other wells with pure media were kept intact for control (see figure 2 in appendix 3). Pictures were taken every 10 minutes for the first half-hour, and every 30 minutes for 3 hours.

3.3.3 N-acetylcysteine

The aim of the experiment was to investigate nanoparticle induced cell death with additional exposure to antioxidant N-acetylcysteine (NAC). Cells were exposed to 1mM NAC solution in growth media.

This experiment was conducted on two plates (plate A and plate B) made the same day from the same cell flask. Confluent non-differentiated 4 days-old cells were used.

Plate A:

The cells were pre-treated with NAC for 1h, 3h and 24h prior to exposure to nanoparticles only.

The culture media was removed and replaced by 1mL of new media (without NAC or NP) for the control and the control with only NP in media, and by a 1mL of a 1mM NAC solution in media for pre-treatment (pt) for the rest of the plate (see figure 3 in appendix 3).

After 1h, the media + NAC was carefully removed from the corresponding wells (see figure 3 in appendix 3) and replaced with a 200µM CuO nanoparticles solution in growth media, or with new pure media in the control wells.

The same procedure was repeated after 3h and 24h for the corresponding wells.

Plate B:

The cells were pre-treated with NAC for 1h and 3h before being simultaneously exposed to nanoparticles and NAC. One group of cells was exposed to NAC and nanoparticles simultaneously without any pre-treatment.
The culture media of all the wells in plate B was removed cautiously, and replaced by 1mL of a 200µM CuO NP + 1mM NAC solution in media for one of the groups and by a 1mL of a 1mM NAC solution in media for pre-treatment for the rest of the plate (see figure 4 in appendix 3).

After 1h, the media + NAC was carefully removed from the corresponding wells and replaced with a 200µM CuO nanoparticles + 1mM NAC solution in growth media. The same procedure was repeated after 3h for the corresponding wells (see figure 4 in appendix 3).

Pictures were taken for both plates after 0h, 1h, 24h, 48h and 72h of exposure to the nanoparticles.

### 3.3.4 Limited time exposure to nanoparticles in media

The purpose of this experiment was to investigate the reversibility of CuO NP toxicity with the different times of the exposure to nanoparticles.

Experiment was conducted on cells grown for 4 days with 1mL of media in a 24 well plate and forming a confluent layer at the bottom of the plate. The media was carefully removed from the wells, and replaced by 1mL of the 200µM CuO NP solution or growth media as shown in Figure 5 and Figure 6 in appendix 3.

After the indicated time (10 min, 30 min, 1 h and 3h), the media with NP was carefully removed. For the experiment with exposure to NP for 1h and 3h (figure 5 in appendix 3), media with NP was replaced by new media (without NP). Pictures were taken 0, 1, 2, 3, and 24h after the exposure. For exposure to NP for 10mins, 30mins, 1h (figure 6 in appendix 3) each well was washed three times with new media and finally replaced with media without nanoparticles. Pictures were taken after 0, 1, 3, 6, 24 and 48h of exposure to NP.

### 3.3.5 Limited time exposure to nanoparticles in Ringer’s solution

The experiment was performed in order to test the interactions between cells and nanoparticles at different pH values. Cells in a 24-well plate were exposed to 200µM of CuO NP in Ringer’s solutions with pH7.3 and pH5 (prepared from RS pH 7.3; pH adjusted with
1M HCl solution). Wells with culture media only, 1h exposure to Ringer’s solutions at pH 5 and pH 7.3 and media with NP were made as control groups. (Figure 7 in appendix 3)

The experiment was performed on cells grown for 4 days in a 24 well plate and forming a confluent and differentiated layer.

In the experiment, the media was carefully removed from the wells and the plates were filled in as show in figure 7 in appendix 3.

After one hour of exposure, the Ringer’s solution with NP at pH 5 and pH 7.3 was carefully removed and the wells were washed three times with RS at pH 5 and pH 7.3 respectively. Then Ringer’s solution was replaced with media. For the control groups exposed to only Ringer’s solution at pH 5 and pH 7.3, new media was added without washing. Wells exposed to NP in media for 1 hour were washed 3 times with media and then new media was added. Pictures were taken after 0, 1, 2, 3, 4, 24, 48 and 96 hours of exposure to NP.

### 3.3.6 Determination of time related cytotoxicity of nanoparticles

The purpose of the experiment was to determine the time needed for the toxic effect of NP to develop. It was performed on newly confluent, non-differentiated cells grown for 4 days in 24-well plate. Cells were exposed to 200µM of CuO NP solution in growth media and observed for 24 hours with pictures taken every 1 hour.

### 3.3.7 Endocytosis inhibition with cytochalasin D

The experiment was performed to test the nanoparticle uptake mechanism. Cytochalasin D was used to inhibit the formation of actin filaments and therefore prevent endocytosis.

The experiment was performed on newly confluent, non-differentiated cells grown for 4 days.

A6 cells were exposed to 10µM cytochalasin D (in growth media) for 1h and 5µM cytochalasin D for 10 min and 30 min. Then cytochalasin D solutions were carefully removed and 200µM NP solution was added. For control group, after 10 min and 30 min exposure,
cytochalasin D solution was replaced by growth media. Control groups with only media and 200µM NP solution in media were also included (Figure 8 in appendix 3).

3.3.8 Fluorescence-activated cell sorting analysis of dividing cells exposed to nanoparticles

The purpose of the experiment was to determine how exposure to CuO NP would affect the cell cycle of dividing A6 cells.

80% confluent non-differentiated A6 cells were exposed to 200µM CuO NP in a culture flask, while having a control group with replaced media. Cells were prepared for fluorescence-activated cell sorting (FACS) analysis with flow cytometer (see Appendix 6) 24 and 48 hours after addition of NP / change of media.

The following procedure was followed for each flask:

80% confluent A6 cells grown in a culture flask were trypsinized and 6ml PBS were added. The cell suspension was divided into two FACS tubes and centrifuged for 5 minutes at 900rpm (corresponding to 200 g) at room temperature. The supernatant was discarded and 3 ml PBS were added. Then, the cell suspension was again centrifuged for 5 minutes, the supernatant was discarded and the cells were re-suspended in 3 ml PBS.

The concentration of cells in 3ml PBS was determined using Bürker-Türk counting chamber, then the amount of the solution containing 500,000 cells was transferred to a new FACS tube and PBS was added to a total volume of 3ml. The tube was centrifuged at 900rpm. Supernatant was discarded and the cells were re-suspended with 200µl PBS. Then 2 ml of ice-cold 70% ethanol were added while whirlmixing. The sample was incubated on ice for 30 min and then stored at -20°C.

Prior to FACS analysis, the A6 cells were stained with propidium iodide which binds to the DNA and RNA. Before staining, the cells were centrifuged at 1200 rpm (300 g) for 5 min at 4°C. Supernatant was discarded, then 2ml of PBS were added and the sample was centrifuged again at 1200 rpm. Then, 400µl PBS, 50µl RNase (to degrade RNA molecules) and 50µL propidium iodide were added and the cells were incubated for 30 min in a tube covered with tin foil.
In FACS analysis, 10,000 cells were analyzed per sample. The fluorescence of each individual nucleus as well as forward scatter (FSC) and side scatter (SSC) of each cell were measured. Data was collected and analyzed with FACSAn software (cell quest pro and mod fit). The following settings of flow cytometer were used: 488nm argon laser light beam, 560nm dichrom mirror, 600nm band pass filter was used.

### 3.4 Pictures analysis

During the experiment, cells were observed through the inverted light microscope LEICA DMI R B at 100 times magnification and pictures were taken. In every experiment pictures taken 5-10 minutes after the beginning of exposure are referred to as t0 pictures.

In our study acute toxicity was defined as the effect that causes severe symptoms that advance rapidly on the cells and cause cell death within few hours (up to 3h) from the exposure.

To obtain quantitative results of the amount of dead cells, pictures were analyzed with Image J image processing program. First, each image was converted to an 8-bit image (black and white) (figure 24).

![Figure 24: Left: usual cell culture picture, right: black and white cell culture picture obtained using ImageJ](image)

Then by adjusting the threshold only white cells are visualized on the picture. These white-looking cells will be called “white dying cells”, they consist of dead or dying cells, as well are cells that have loosened from the bottom of the flask. The domes and balloons formed by the cells are also appearing white in the program. The percentage of black area is measured, the percentage of white dying cells being 100%-% black area (figure 25).
**Figure 25:** Cell monolayer picture with dead cells in white and dark are measured (% Area). For example, the figure shows 90.03% of black area, which means the percentage of white dying cells is 100 - 90.03 = 9.97%.

The percentages of white dying cells were obtained from ImageJ were then entered into GraphPad Prism 5 and plugged into graphs. Statistical analyses of the results were also made using this program: t-tests and two-way ANOVA tests (Appendix 2).
4.1 Copper oxide nanoparticles and copper ions toxicity on a confluent cell layer

CuO NP can be clearly seen and appear as black-brown dots on the cell monolayer. The results show that CuO NP and Cu$^{2+}$ ions do not have an acute toxic effect on A6 cell line, since no immediate effect was observed upon exposure (figure 26).

However, compared to the control group (figure 26), CuO NP at 200µM concentration, begin to induce cell death already in 6 hours after exposure. Great amounts of dead cells can be observed after 24 and 48 hours, with almost no living cells left after 72 hours.

Results for the lower concentration (20µM and 2µM CuO NP and Cu$^{2+}$) are in appendix 4.

By graphing the obtained percentages of dead cells at each time of exposure, it can be seen that the toxic effect of CuO NP appears to be dose dependent (see figure 27). It appears
that 6 hours are needed for development of clear toxic effect with 200µM and 20µM CuO NP. However, 24h hours are needed for the expression of the clear toxic effect with 2µM.

Figure 27: Percentage of white dying cells for the exposure to 200, 20 and 2µM CuO NP. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).

A two-way ANOVA analysis of the data in figure 27 confirms that the toxicity of nanoparticles is both time and concentration dependent, as the analysis considers the effects of both concentration and time to be extremely significant (P < 0.0001).

By analyzing the effect of Cu²⁺ ions on A6 cells (see figure 2 and appendix 4), it is clear that Cu²⁺ ions are less toxic to the cells, since the highest amount of dead cells at the highest concentration is only 35% after 96 hours, whereas for exposure to NP amount of dead cells is close to 100% at 96 hours. Moreover, at a similar (200µM) concentration than the NP, copper ions do significantly affect the cells after 6h, but only after 24h of exposure (Figure 28).
Figure 28: Percentage of white dying cells for the exposure to 200, 20 and 2 µM Cu^{2+}. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).

A two-way ANOVA analysis of the data in figure 28 confirms that the toxicity of copper ions is both time and concentration dependent, as the analysis considers the effects of both concentration and time to be extremely significant (P < 0.0001).

4.2 Acute toxicity of copper oxide nanoparticles on non-differentiated single cells

Copper oxide nanoparticles can be clearly seen. They emerge as black-brown spots on the cell monolayer. The results show that CuO NP do not have an acute toxic effect on A6 cell line, since no immediate effect was observed upon exposure and throughout the first 3 hours of the experiment (Figure 29).

Other pictures taken every 10 mins for the first 30 mins and every 30 mins for the following 3 hours between 0 and 3h after exposure to 200µM CuO NP are shown in figures 3 and 4 in appendix 4.
After 22 hours, the wells with cells containing nanoparticles showed the massive cell death confirming the toxicity of the nanoparticles on the cells (Figure 30).

Figure 29: Pictures of A6 cells exposed to 200 µM NP taken during the first 3 hours of exposure and control group.

Figure 30: Pictures of A6 cells exposed to 200 µM CuO NP and control (in media) 10 min after beginning of exposure and 22 hours of exposure.
4.3 N-acetylcysteine

From this experiment, we can first of all show that N-acetyl-L-cysteine does not have a toxic or lethal effect on the cells. It is clear by looking at figure 31 that the wells in which cells were exposed to NAC for up to 72h do not present more dead cells than in the control group without exposure to NAC, or nanoparticles. The white aggregates visible on the pictures do not represent dead cells, they are balloons.

Control without NAC exposure:

Control with NAC exposure:

Figure 31: Pictures of A6 cells exposed to media only, and to NAC (in media), from 10 min to 72 hours of exposure to NAC (1mM).

1h pre-treatment:

When looking at the pictures (figure 32) of the cells which were exposed to NAC only during the pre-treatment, we can observe only a very slight improvement of cell survival. However, there is no improvement visible at all for the cells exposed to NAC both during the pre-treatment and the exposure to NP.
**Figure 32:** Cells pre-treated with NAC (1mM) for 1h before exposure to NP only or NP and NAC. Pictures taken 0, 1, 24, 48 and 72h after addition of 200µM CuO NP.

The graph in figure 33 shows that there are minor improvements in cell survival (just below 10%) after 24h, for the cells which have been exposed to NAC only during the pre-treatment. However, it seems that NAC does not have any effect on the cells exposed to NAC both during pre-treatment and during nanoparticles exposure.
Figure 33: Percentage of white dying cells for 1h NAC pre-treatment followed by exposure to NP only and NP + NAC. Concentrations: 1mM NAC, 200µM CuO NP. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).

3h pre-treatment:

Only a slight delay in cell death induction is visible after 24h for the cells exposed only to nanoparticles after the 3h NAC pre-treatment. However, the cells exposed to NAC continuously (NAC and NP after 3h) show a significant improvement of cell survival in comparison to the control group after 24h, see figure 34 and 35.
Figure 34: Cells pre-treated with NAC for 3h before exposure to NP only or NP and NAC Concentrations: 1mM NAC, 200µM CuO NP. Pictures were taken 0, 1, 24, 48, 72h after NP exposure.

The noticeable improvement of cell survival seen in figure 34 for the cells exposed to both NAC and NP after 3h NAC pre-treatment is confirmed by the graph obtained from the picture analysis with the imageJ program in figure 35.

Figure 35 also validates our argument on a slight delay in cell death induction for cells pre-treated with NAC prior to exposure to NP only; however, this delay is not significant.
We can affirm from this part of the experiment that the use of NAC as pre-treatment for 3h before exposure to NP only has a minor delaying effect on cell death induction. Moreover, we have shown that a continuous exposure to NAC following a 3h pre-treatment induces a significant delay in the cells’ death until sometime between 24h and 48h. However it seems that this effect is only temporary as the percentage of dead cells in both of those cases reaches the control group percentage after only 48h (see figure 35). Therefore, we can affirm that a 3h pre-treatment with NAC does not inhibit the cell death induction.

24h pre-treatment:

Figure 36 shows that with a 24h NAC pre-treatment prior to exposure to NP, there is a very slight delay in cell death for at least 48h.
From the graph in figure 37 we can assess that a 24h NAC pre-treatment enables some of the cells to survive for a time significantly longer (between 48h and 72h) than they could do without any NAC exposure.

**Figure 36**: Cells pre-treated for 24h with NAC (1mM) before exposure to 200µM CuO NP.

**Figure 37**: Percentage of white dying cells for 24h NAC pre-treatment followed by exposure to nanoparticles. Concentrations: 1mM NAC, 200µM CuO NP. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).

**No pre-treatment:**

In figure 38, we can observe a slightly higher number of living cells after 48h in the group of cells exposed simultaneously to NAC and nanoparticles (without any pre-treatment) than in the control group.
Figure 38: Cells exposed simultaneously to NAC + NP without NAC pre-treatment only show a delay in cell
death induction after 48h. Concentrations: 1mM NAC, 200µM CuO NP.

Even though the analysis of the cell pictures in figure 39 does not show any significant
delay in cell death induction after 24h, we can observe that the percentage of dead cells after
48h is about 10% lower for the cells exposed to NAC than for the control group. However, at
72h, we can see that both groups of cells express a similar dead cell percentage, which
confirms again that NAC does not inhibit cell death induction, but only postpones it.

Figure 39: Percentage of white dying cells for exposure to NAC simultaneously without pre-treatment.
Concentrations: 1mM NAC, 200µM CuO NP. Bars represent the mean ± SE. An * indicates a significant
difference from the control NP group (P < 0.05).
4.4 Limited time exposure to nanoparticles in media

The aim of the experiment was to remove CuO NP by washing the wells and replacing the growth media. However, nanoparticles could still be observed after this procedure when the cells were exposed to nanoparticles for 1 and 3h (Figure 40).

Therefore, we decided to repeat the experiment with shorter exposure time, in order to have a more efficient removal of the nanoparticles, since they would have less time to interact with the cells’ surface. The smaller aggregates of nanoparticles could be successfully washed away, unlike the larger ones, which remained attached to the surface of the cells (figure 41).
After 24 hours cell death was observed in both NP control group and the wells were NP were washed. However the amount of the dead cells was not the same: cells were more affected by NP in the wells where the NP were not washed. In the wells exposed to NP for the shortest period (10 min) number of dead cells was lower than in wells exposed for 1 hour (Figure 42).
After a 24h exposure to NP:

![Images showing A6 cells exposed to 200 µM NP for 10, 30 min and 1 hour before washing with media, control NP – cells exposed to NP and not washed. Pictures obtained 24 hours after addition of NP.]

**Figure 42:** A6 cells exposed to 200 µM NP for 10, 30 min and 1 hour before washing with media, control NP – cells exposed to NP and not washed. Pictures obtained 24 hours after addition of NP.

After 48 hours, almost no living cells were observed in the control group exposed to NP without washing, whereas wells washed with media had a relatively large amount of living cells (Figure 43). Generally it appears that less NP can be washed away after a prolonged exposure time, thus resulting in larger amount of NP remaining. This leads to increase in cell death.

After a 48h exposure to NP:

![Images showing A6 cells exposed to 200 µM NP for 10, 30 min and 1 hours before washing with media; control NP – cells exposed to 200 µM NP without washing. Pictures obtained 48 hours after addition of NP.]

**Figure 43:** A6 cells exposed to 200 µM NP for 10, 30 min and 1 hours before washing with media; control NP – cells exposed to 200 µM NP without washing. Pictures obtained 48 hours after addition of NP.
4.5 Limited time exposure to nanoparticles in Ringer’s solution

After careful removal of the Ringer’s solution with NP and washing of the wells, nearly all NP seemed to be removed (Figure 44 top row).

![Figure 44: Pulse experiment to NP in Ringer’s solution with pH 5 and pH 7.3. Pictures taken 1 hour after addition of 200µM NP (after washing the NP and replacing Ringer’s solutions); 1 hour after change of media for control – media group.](image)

There has been no increase in the cell death after limited time exposure to Ringer’s solution without NP at both pH 5 and pH 7.3.

After 48 hours it was confirmed that RS with different pH values improves the amount of NP that are washed away. The closer examination of the cells exposed to NP for 1h in Ringer’s solution and afterwards washed, showed the lower cell death rate in comparison to the control wells with nanoparticles in media (Figure 45).
Quantitative analysis of the amounts of dead cells also shows that there is an improvement in survival rate after 24 and 48 hours for the cells washed with Ringer’s solution (Figure 46): a greater improvement was observed for the cells treated with RS pH 5.

Figure 45: Pulse experiment to NP in Ringer’s solution with pH 5 and pH 7.3. Pictures taken 48 hours from the beginning of exposure to 200µM NP; 48 hours after change of media for Control –Media group. Groups NP with RS pH 5 and NP with RS pH 7.3, RS pH 5 and RS pH 7.3 have been washed with RS and replaced with media 1 hour after addition of NP

Figure 46: Amount of white dying cells of the groups incubated with NP and washed with Ringer’s solution pH 5 and pH 7.3; media with NP (Control NP); media only (Control). Concentration: 200µM CuO NP. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).
4.6 Determination of time related cytotoxicity of nanoparticles

By analyzing pictures with Image J picture analyzer and quantifying the amount of white dying cells, it can be clearly seen that toxic effect of NP begins to manifest 7-9 hours after the addition on NP: the number of dead cells increases from ~5% to 15% (see Figure 47). Even though there seems to be a decrease in the percentage of dead cells at 13 hours, the amount of dead cells is generally gradually increasing and reaches 40% at 24 hours.

![Graph showing percentage of white dying cells over time](image)

**Figure 47:** Percentage of white dying cells within 24 hours of exposure to 200 µM CuO NP solution. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).

Decrease in percent of dead cells at 13-15 hours can be observed. However, this decrease can be explained by the fact that dead A6 cells often detach from the bottom of the plate and therefore cannot be easily focused on with the microscope.

4.7 Endocytosis inhibition with cytochalasin D

The cells pre-treated with 10µM CD for 1h appeared white and seemed to loosen from the bottom of the wells only 1h after the 1h CD pre-treatment. And 2h after the pre-treatment the cells were detached from the bottom of the well. Therefore, we repeated the experiment with a lower CD concentration (5µM), and shorter times of exposure (10 and 30mins).

It can be observed on the pictures in figure 48 that cytochalasin D has a reversible effect on the cells: only a few minutes after exposure, the cells seem to be detaching from the substrate and forming “arm-like” extensions. However, these features disappeared.
progressively, leaving intact cells a few hours after replacement of the media supplemented with CD by a pure media (figure 48). A 5µM cytochalasin D solution did not show any visible lethal effect on the cells (figure 49).

**Figure 48:** Effect of cytochalasin D on the cells. 30 minutes after exposure to 5µM CD the cells exhibit a formation of “arm-like” structures, which have completely disappeared 2h after replacement of CD with new media.

**Figure 49:** Picture of A6 cells pretreated with 5µM Cytochalasin D for 10 and 30 min and control group with replaced media (no NP or CD). Pictures taken at 0 (=5min), 2, 10, 24, 48 hours after change of media / replacement of CD. The white aggregates visible on the pictures do not represent dead cells, they are balloons.
On the cells pre-treated for 10 and 30 minutes with CD prior to NP exposure, the cell death induction was significantly lowered after a 24h exposure to NP (figures 50 and 51). The cells also returned to their regular morphological appearance a few hours (~2h) after removal of the CD. The inhibition of the nanoparticle uptake, and therefore the survival rate of the cells seem to be more pronounced when the cells were exposed to cytochalasin D for 30 mins instead of 10 mins (figure 51).

Figure 50: Pictures of A6 cells pre-treated with 5µM Cytochalasin D for 10 and 30 min before addition of 200 µM NP and control group with NP (without any pre-treatments). Pictures taken at 0 (±5 min), 2, 10, 24, 48 hours after addition of NP.
Figure 51: Percentage of white dying cells after up to 48h exposure to NP following a 10mins or 30mins pre-treatment with cytochalasin D. Concentrations: 5µM CD, 200µM CuO NP. Bars represent the mean ± SE. An * indicates a significant difference from the control NP group (P < 0.05).

4.8 Fluorescence-activated cell sorting analysis of dividing cells exposed to nanoparticles

By measuring fluorescence of propidium iodide, the amount of DNA in each individual cell was determined. Using FACS analysis software data was analyzed and amount of cells in G_0/G_1, S, G_2/M phase and apoptosis was determined (Figure 52).
Figure 52: FACS analysis for control group of A6 cells grown for 5 days in media: large peak at 70-95 represents cells in G₁ phase, peak at 100-125 corresponds to cells in G₂ phase.

It can be seen from figure 52 that A6 cells not exposed to NP were present in all phases of cell cycle, with G₀/G₁ phase prevailing. The rate of apoptosis was very low. Cells exposed to NP for 24 hours (figure 53) were mostly in G₀/G₁ and S phases, whereas G₂/M was generally absent. The amount of apoptosis was higher compared to the control group.
From the quantitative analysis of the data from FACS analysis (figure 54) it can be clearly seen that there is no significant increase in apoptosis with cells exposed to NP for 24 hours. The amount of cells in G₀/G₁ phase is nearly the same for the exposed cells and the control. However, there is a significant difference between percentage of cells in S and G₂/M phases. A very small amount of cells were in G₂/M phase compared to the control, whereas the amount of cells in S phase significantly increased with exposure to NP.
Figure 54: % of measured cells in G0/G1, S, G2/M phases and apoptosis 24 hours after exposure to 200µM CuO NP (change of media for control). Bars represent the mean ±SE. An * indicates significant difference to from the control group (P<0.05).

48 hours after the beginning of the experiment, cells in the control group were still present in all the phases of cell cycle (figure 56), and the degree of apoptosis was still very low. Cells exposed to NP for 48 hours (figure 57) were present in G0/G1 and S phases, whereas G2/M phase appeared to be completely absent. The rate of apoptosis seemed to be greatly increased compared to 24 hour exposure and the control.
Figure 56: FACS analysis of the control group 48 hours after beginning of the experiment. Peak at 70-85 – 
$G_0/G_1$ phase, peak at 95-125 – $G_2/M$ phase.

Figure 57: FACS analysis of A6 cells exposed to 200 µM CuO NP for 48 hours. Peak at 75-100 represents cells 
in $G_0/G_1$ phase, $G_2/M$ phase is not observed.
By quantitative analysis it is clearly seen that there is a significant increase in apoptosis 48 hours after the exposure to NP compared to with the control (figure 58). Also it appears that there is no considerable alteration in the number of cells in $G_0/G_1$ phase. The variance of cells in $S$ and $G_2/M$ phases is of clear significance between exposure to NP and the control group: the percentage of cells in $S$ phase is much higher for cells exposed to NP, whereas the amount of cells in $G_2/M$ phase is much lower.

**Figure 58:** Percentage of measured cells in $G_0/G_1$, $S$, $G_2/M$ phases and apoptosis 48 hours after exposure to 200µM CuO NP (change of media for control). Bars represent the mean ±SE of 2-3 values. An * indicates significant difference to from the control group ($P < 0.05$).

FACS analysis of the size of the cells shows an increase in the number of small cells 24 and more clearly 48 hours after the addition of nanoparticles (figure 59). Such small-sized cells are cells in apoptosis. In control group there seems to be no such an increase (figure 59).
Figure 59: FACS analysis of cell size using forward light scatter. Cell size is plotted against the number of cells for control group (24 and 48 hours after beginning of the experiment) and exposure group (24 and 48 hour exposure to 200µM CuO NP)

4.9 Effect of nanoparticles on differentiated and non-differentiated cells

In nearly all of our experiments, control groups with exposure to 200 µM NP were included as a reference of the cell death rate after 24 and 48 hours of exposure. In some of the experiments cell death was measured for the exposure of newly confluent cells, whereas in other differentiated cells were used. When comparing those two types of cells, it appears from our experiments that the vulnerability of cells depends on their age and differentiation. Figure 60 shows that a significantly lower amount of differentiated cells die after 24-hour exposure
to NP. After 48-hour exposure clearly more differentiated cells die. Differentiated cells are all in a G\(_0\) phase, whereas the non-differentiated cells are present in all phases of cell cycle.

**Figure 60:** Death rates of non-differentiated and differentiated A6 cells 0 (=5min), 24 and 48 hours of exposure to 200 µM CuO NP. Bars represent the mean ± SE. An * indicates a significant difference from the non-differentiated cell group (P < 0.05).
5. Discussion

When exposing cells to nanoparticles, the resulting exposure concentration appears to be higher than intended since nanoparticles do not stay distributed in the solution, but settle down directly on the cells. This is especially important when comparing the toxic effect of NP to that of Cu$^{2+}$ ions, since ions stay in the solution. Presence of CuO NP outside and inside the cells may cause a local increase in concentration of Cu$^{2+}$; therefore, exposure to Cu$^{2+}$ solutions was investigated in our study. However, the concentrations of Cu$^{2+}$ that were used did not have such an extensive effect as the corresponding concentrations of NP. If nanoparticle-induced cell death is triggered by excessive amounts of copper ions inside or outside the cells, this suggests that the concentration of Cu$^{2+}$ ions liberated from NP might be higher than the ones used in our study. It is also probable that Cu$^{2+}$ liberated from NP directly inside the cell can reach the target structures in the cell and cause damage more easily than Cu$^{2+}$ present outside of the cell in growth media. The impact of Cu$^{2+}$ could be tested with exposure to higher concentrations of CuCl$_2$. Moreover, the liberation of copper ions increases at lower pH, so there should have been an increased amount of Cu$^{2+}$ upon 1 hour exposure to NP in Ringer solution pH 5. However even if such an increase took place, it was reduced after washing of the wells and replacement with media.

In general, the concentration of NP that was used in our study (200µM) appears to be too high for any additional treatment to completely prevent cell death. Therefore, a positive effect could only be observed as a delay in cell death.

There appears to be a specific interaction between the surface of the cells and NP, since they seem to stick to the apical membrane of the cells; therefore, even washing the cells several times with media did not significantly remove the nanoparticles. We might suggest that NP attach to the surface of the cells through interactions with glycocalyx due to the negative charge of sialic acid present on it (Alberts et al., 2007). Therefore, it is possible that this attraction could be influenced by changing the H$^+$ concentration of the solution. In our experiment, when Ringer’s solution at pH 5 and 7.3 were used for NP suspension, more NP could be successfully removed from the cell surface. Thus, a greater cell survival was observed after up to 48 hours of exposure.
It appears that 7-9 hours are needed for development of a clear toxic effect of NP (5-17% death cells), which may be due to the accumulation of NP in the cell. In this context, it can be seen from our results that 7-9 hours are needed for the cells to take up a significant amount of NP and for the damage caused by NP to accumulate inside the cells. However, if we assume that all the cells in the culture had the same NP uptake mechanism, then NP should have caused an extensive cell death in all the culture after 7-9 hours of exposure. In our results, a gradual increase of cell death was observed after 7-9 hours, thus it might be suggested that such tendency was due to different sensitivity of A6 cells, since cells in a culture are present in different phases of cell cycle. Also the difference in response of the cells to NP can be explained by uneven distribution of NP in the media and the surface of the cell culture thus making some cells exposed to higher amounts of NP.

Nanoparticles might be causing cell death mainly due to the increased oxidative stress as proposed by Unfried et al. (2007). However, some other toxic mechanisms may be involved since using N-acetyl-L-cysteine as an antioxidant against the oxidative stress induced by nanoparticles only enabled some of the groups of cells to delay the induction of cell death. For the cells exposed to nanoparticle + NAC after a 3h NAC pre-treatment, the most significant improvement could be observed. The other combination of NAC + NP showed no or very little delay in cell death. This might be due to the effect of NAC on the cells’ endogenous antioxidants; and therefore, NAC alone is not sufficient enough to inhibit cell death induction. Since NAC is a precursor for GSH, the cells’ own GSSG reduction mechanism (to GSH) might be down-regulated. And as NADPH is the reducing agent of GSSG, its production is also down-regulated. NADPH is the main source of superoxide, and a reduction in the amount of NADPH would result in a lower production of superoxide; and therefore, in a decrease in SOD synthesis (Guo et al., 2007). Reduced SOD synthesis and reduction of GSSG would weaken cells own defense against NP-induced ROS. The induction of cell death might have been affected if NAC was added at around 5 hours after the addition of NP, before the ROS levels are lethal to the cells. A significant improvement in cell survival would confirm oxidative stress induced cell death.

As stated by Unfried et al. (2007), the damage caused by NP mostly affects the intracellular structures of the cells; therefore, it is most likely that NP are taken up by the cells through endocytosis. When cytochalasin D was introduced to improve the survival of A6 cells, it was most likely that cytochalasin D could only inhibit the endocytosis for a limited amount of time, because it was removed from the media after 10 and 30mins. Morphological
changes due to a disrupted cytoskeleton structure disappeared only a few hours after CD was removed from the media; therefore, it is likely that the endocytosis mechanism started again at that time. This would mean that the cells still took up nanoparticles after a certain period of time and cell death signaling was still triggered. Moreover, the inhibition of endocytosis is not nanoparticle-specific; therefore, some essential organic compounds cannot be taken up by the cells. As cytochalasin D binds to the actin filaments breaking them and inhibiting their growth, the cytoskeleton of the cells is severely damaged and even though microtubules are not affected, this might still affect the internal organization of the cells. Partial disruption of cytoskeleton affects the cell surface: CD also destroys the microridges present on the cells’ surface, may prevent the nanoparticles to stick on the cells’ apical membrane (Schropfer et al., 2000).

Our study shows that there is a difference in sensitivity of differentiated and non-differentiated A6 cells to NP. Differentiated cells seem to be less sensitive in the first 24 hours of exposure. This might be due to the fact that non-differentiated cells are in the cell cycle and perform DNA check; and thus, are probably more likely to sooner detect the DNA damage caused by NP than differentiated cells which are in the $G_0$ phase. After 48 hours of exposure, differentiated cells appear to be significantly more affected by NP. Indeed, these cells have a higher metabolic rate than non-differentiated cells, and thus might produce more ROS or copper ions when interacting with CuO NP. In this context it is probable that differentiated cells in an organism would be more affected by NP in the long run.

Our results also indicate that NP have a different effect on the cells depending on the phase of the cell cycle they are in. It is generally known that cell cycle stops mostly occur at three checkpoints: G1 start, G2/M, M (metaphase-to-anaphase checkpoint) (Alberts et al., 2007). However, as our results indicate, nanoparticles can cause a cell cycle arrest at S phase. Cell cycle stop at S phase and increase in apoptosis have also been demonstrated with exposure of human ovarian granulosa cells (synchronized at the same cell cycle phase) to 100µM calcium phosphate nanoparticles (Liu et al, 2010.). In this study, calcium phosphate NP were shown to enter the cells and accumulate in the membrane compartments, including mitochondria and lysosomes. It has been proposed by Liu et al. that calcium phosphate NP might cause an inhibition of DNA replication or cause impairments in the progression of S phase. We might hypothesize that CuO NP also have a similar effects and can for example interfere with the activation of cyclin-dependent kinases (ckds) or cyclin proteins and especially S cyclins which normally ensure the progression of the S phase.
Even though A6 cells are derived from a distal part of frog kidney, they can be used as a general representation of tight epithelia in which transport occurs through the cell layer. Therefore, our study can be relevant when discussing effect of nanoparticles on other types of tight epithelia, such as lung epithelia and intestine epithelia. This is especially applicable since primary exposure of an organism to nanoparticles might be through ingestion or inhalation.
6. Conclusion

Copper oxide nanoparticles induce cell death in A6 culture. From our experiments and other studies the following model of events taking place during CuO NP exposure can be created (figure 6.1):

**Figure 6.1:** Effect of copper oxide nanoparticles on the cell and its cellular structures. 1. The actin microfilaments (MF) attach to the curvature in the cell membrane. 2. The actin MF are pushing on the membrane of a forming vesicle and driving it away from the cell membrane (Galletta et al., 2010). 3. The new vesicle contains copper oxide nanoparticles. 4. Lysosome containing degradative enzymes fuses the vesicle with the NP. 5. ROS are produced inside of the lysosome containing NP. 6. Copper ions (liberated from NP) and ROS enter the nucleus and damage the DNA (Unfried et al., 2007). 7. NP interact with mitochondria and increase the production of ROS (Unfried et al., 2007). 8. Cytochrome C released from mitochondria (Alberts et al., 2007) and p53 activation due DNA damage induce apoptosis (Martindale and Holbrook, 2002).
The toxic effect of NP is time and dose dependant, with 7-9 hours needed for the induction of cell death at 200µM CuO NP concentration. Cell survival could be improved with addition of antioxidant and/or inhibition of endocytosis.

Cells exposed to the nanoparticles shrink thus showing the signs of the apoptosis. CuO NP caused cell cycle stop in S phase, but the exact mechanisms need further investigation.
7. Perspectives

As there have not been many studies conducted yet on the effects of copper oxide nanoparticles on epithelial kidney cells, there is a broad range of novel experiments to perform. An interesting subject to investigate is the exact NP uptake mechanism of the cells. The toxicity of different sized nanoparticles could also be tested. As well, the comparison of ROS level in cells exposed to NP and metal ions could be of interest.
8. References

- Barzilai A, Yamamoto KI, DNA damage responses to oxidative stress, 2004. DNA Repair; 3; 1109-1115.
- GraphPad Prism, Fifth Version, 2011.
- Moriwaki H, Osborne MR, Phillips DH, Effects of mixing metal ions on oxidative DNA damage mediated by a Fenton-type reduction, 2008. *Toxicology in Vitro*; 22; 36-44.

WEB REFERENCES:

- Molecular Probes, Propidium Iodide Nucleic Acid Stain, 2006, Invitrogen http://www.invitrogen.com (on the web 2011-05-20);
- Sigma Aldrich: www.sigmaaldrich.com (on the web 23-03-2011)
- UpToDate http://www.uptodate.com/contents/image?imageKey=NEPH/14450 (on the web 23-03-2011)
9. Abbreviations and definitions

- Apaf-1: Apoptotic Protease-Activating Factor-1
- Apoptosis: programmed cell death
- CD: Cytochalasin D
- Cdk: Cyclin-dependent kinases
- Centrosome: major microtubule-organizing center
- CME: Clathrin-Mediated Endocytosis
- CuO: Copper (II) Oxide
- FACS: Fluorescence-Activated Cell Sorting
- FSC: Forward Scatter
- GSH: glutathione
- GSSG: glutathione disulfide (oxidized form of GSH)
- H$_2$O$_2$: hydrogen peroxide
- MF: MicroFilaments
- mtDNA: mitochondrial DNA
- NAC: N-acetylcysteine
- NAD$: Nicotinamide Adenine Dinucleotide
- NADPH Oxidase: membrane-bound enzyme complex, which can be found in the plasma membrane as well as in the membrane of phagosome.
- NADPH: reduced form of NAD$^+$ (Nicotinamide adenine dinucleotide phosphate)
- NOX: NADPH oxidase
- NP: NanoParticles
- O$_2^·$: superoxide
- ‘OH: hydroxyl free radical
- PARP: Poly (ADP-Ribose) Polymerase
- pt: pre-treatment
- ROS: Reactive Oxygen Species
- RS: Ringer’s Solution
- SE: Standard Error
- Senescence: aging
- SOD: SuperOxide Dismutase
- SSC: Side Scatter
## 10. Appendix

### Appendix 1: DMEM composition

**Table 1: DMEM composition (Invitrogen.com):**

<table>
<thead>
<tr>
<th>COMPONENTS</th>
<th>Molecular Weight</th>
<th>Concentration (mg/L)</th>
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Appendix 2: T-test and p-value

A t-test is used to compare the means of two groups of data and investigate the likelihood that the difference observed between those two means is not due to “chance”. First a null hypothesis has to be stated, we could say that this hypothesis is the opposite of what we actually want to show. For instance when comparing a control group (with cells exposed to media only) to cells exposed to 200µM NP, our null hypothesis would be “Cells exposed to 200µM NP do not exhibit a higher percentage of dead cells than the control group”. We therefore try to reject this null hypothesis by using statistics. In order to do so, a p value is calculated; this value is a probability; and thus, its value ranges from 0 to 1 (0 to 100%). The most commonly used confidence interval is 95%, it is the interval between the difference of our two means, and the p value. Therefore, if the p value is lower than 0.05 (5%), then our null hypothesis is false, meaning that there actually exists a significant difference between the means of our two groups of data. And a p value larger than 0.05 indicates that there is no proof that the two means really differ (GraphPad Prism, 2011).

There exist two kinds of p value, one-tailed and two-tailed. When using the one tailed t-test, the tendency of the results should be known or predictable from previous experiments. Whereas, if such a tendency cannot be predicted, a two tailed test should be used (GraphPad Prism, 2011).

The two-way ANOVA test allows investigating how a response is affected by two changing factors (GraphPad Prism, 2011). For example, we examined the effect of both time and concentration on the cell’s response to NP toxicity.
Appendix 3: Plate arrangements

Figures 70 to 77 in this appendix are the plate arrangement for the experiment 1, 2, 3, 4, 5, and 7. In all of the figures, “Control” indicates that the cells are exposed to a new “pure” media. The “Control NP” wells were exposed only to CuO nanoparticles at a concentration of 200µM. The groups which have been pre-treated before exposure to NP (or any other chemical) are designated the following way: for instance “NAC 3h pt + NP and NAC” indicates that the cells were pre-treated with NAC for 3h before exposure to both NP and NAC simultaneously.

Copper oxide nanoparticles and copper ions toxicity on a confluent cell layer:

![Figure 1: 24 well plate arrangement with control group and different concentrations of CuO NP and copper ions.](image-url)
Acute toxicity of copper oxide nanoparticles on non-differentiated single cells:

**Figure 2**: Plate arrangement for the acute toxicity experiment: control group and wells exposed to 200µM CuO NP.

**N-acetylcysteine**:

**Figure 3**: Plate A arrangement for the NAC pre-treatment experiment. Control groups with media only, media and NP, and exposure to NAC only for 1.3 and 24h. Wells exposed to NP only after 1, 3, and 24h pre-treatment with NAC. Concentrations: 1mM NAC, 200µM CuO NP.
Figure 4: Plate B arrangement for the NAC pre-treatment experiment. Control group exposed to NAC for 24h, and groups exposed to both NP and NAC simultaneously after 1 and 3h pre-treatment. And group exposed to both NAC and NP simultaneously without pre-treatment. Concentrations: 1mM NAC, 200µM CuO NP.

Limited time exposure to nanoparticles in media:

Figure 5: Plate arrangement for the limited time exposure experiment. Control group with only media, and with continuous 200µM CuO NP exposure (control NP). And groups exposed to 200µM NP for 1 and 3h.
**Figure 6:** Plate arrangement for the limited time exposure experiment. Control group with only media, and with continuous 200µM CuO NP exposure (control NP). And groups exposed to 200µM NP for 1 and 3h.

**Limited time exposure to nanoparticles in Ringer’s solution:**

**Figure 7:** Plate arrangement for exposure to NP in Ringer’s solution. Control groups for NP and media, only and RS at pH5 and 7.3 for 1h. Groups exposed to NP for 1h in media, RS pH5 and RS pH7.3. Concentrations: 200µM CuO NP.
Endocytosis inhibition with cytochalasin D:

**Figure 8**: Plate arrangement for the endocytosis inhibition with CD experiment. Control, Control NP groups, and control groups for CD (10 and 30mins exposure to CD). And groups pre-treated with CD for 10 and 30mins before exposure to NP only. Concentrations: 5µM CD, 200µM CuO NP.
Appendix 4: Results

Copper oxide nanoparticles and copper ions toxicity on a confluent cell layer

Exposure to 20μM CuO NP:

Figure 1: Exposure of A6 cells to 20μM CuO NP and 20μM CuCl₂, control group - changed media. Pictures taken at 0 (=10min), 3, 6, 24, 48 hours.

Exposure to 20μM Cu²⁺:

Control:

Figure 2: Exposure of A6 cells to 2μM CuO NP and 2μM CuCl₂, control group - changed media. Pictures taken at 0 (=10min), 3, 6, 24, 48 hours.
Acute toxicity of copper oxide nanoparticles on non-differentiated single cells

**Figure 3:** Control group - changed media. Pictures taken at 0 (=5min), 10, 20, 30mins, 1, 1.5, 2, 2.5 and 3 hours.

**Figure 4:** Exposure of A6 cells to 200µM CuO NP. Pictures taken at 0 (=5min), 10, 20, 30mins, 1, 1.5, 2, 2.5 and 3 hours.
Appendix 5: Safety sheets

Figure 1: Safety sheet for N-acetyl-L-cysteine
Figure 2: Safety sheet for Cytochalasin D
Appendix 6: Flow cytometry/Fluorescence-activated cell sorting (FACS)

Flow cytometry is based on the analysis of individual particles (cells). When a sample is injected into a flow cytometer, single cells pass through a light beam of a specific wavelength. As the cells pass through the light beam, front scatter (FSC) and side scatter (SSC) of light as well as fluorescence are measured. FSC is used to determine the size of the cell and SSC gives information about the granular content in the cell (Rahman M. 2006).

In our experiment, the cells were stained with a fluorescent compound propidium iodide which binds to DNA and RNA. RNAs were degraded by RNases, therefore propidium iodide only bound to DNA. When bound to DNA, fluorescence of propidium iodide is greatly enhanced (Molecular Probes, 2006). As the cells pass the light beam, fluorescence of propidium iodide was measured. The amount of fluorescence of each cell is proportional to the amount of DNA; and therefore, it is used to determine the phase of cell cycle (Rahman M. 2006). Cells in G₀ and G₁ phases contain the smallest amount of DNA compared to the other phases of cell cycle, therefore the first peak corresponds to the cells in G₀/G₁ phase (figure 1). Cells in S phase start to synthesize DNA, therefore they contain more DNA than G₀/G₁ phase. In G₂/M phases, cells contain the largest amount of DNA (since DNA is already fully replicated); and therefore, they appear farthest to the right in the graph. Cells with a very low amount of DNA (0-150 in figure 1) are apoptotic bodies with fragmented DNA.

Figure 1: Cell cycle analysis graph obtained with FACS analysis. Amount of DNA is plotted against cell count. (SciELO)