Toxicology 291 (2012) 65–72

Contents lists available at SciVerse ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells

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ARTICLE INFO

Article history:
Received 29 September 2011
Received in revised form 28 October 2011
Accepted 29 October 2011
Available online 6 November 2011

Keywords:
Titanium dioxide nanoparticles
Cellular metabolic activity
Cell viability
DNA damage
Comet assay

ABSTRACT

Serious concerns have been expressed about potential risks of engineered nanoparticles. Regulatory health risk assessment of such particles has become mandatory for the safe use of nanomaterials in consumer products and medicines; including the potential effects on reproduction and fertility, are relevant for this risk evaluation. In this study, we examined effects of silver particles of nano- (20 nm) and submicron- (200 nm) size, and titanium dioxide nanoparticles (TiO_2-NPs; 21 nm), with emphasis on reproductive cellular- and genotoxicity. Ntera2 (NT2, human testicular embryonic carcinoma cell line), and primary testicular cells from C57BL6 mice of wild type (WT) and 8-oxoguanine DNA glycosylase knock-out (KO, mOgg1$^{-/-}$) genotype were exposed to the particles. The latter mimics the repair status of human testicular cells vs oxidative damage and is thus a suitable model for human male reproductive toxicity studies. The results suggest that silver nano- and submicron-particles (AgNPs) are more cytotoxic and cytostatic compared to TiO_2-NPs, causing apoptosis, necrosis and decreased proliferation in a concentration- and time-dependent manner. The 200 nm AgNPs in particular appeared to cause a concentration-dependent increase in DNA-strand breaks in NT2 cells, whereas the latter response did not seem to occur with respect to oxidative purine base damage analysed with any of the particles tested.

1. Introduction

The dramatic expansion of the nanotechnology industry has prompted the need to investigate potential toxic effects of nano-sized particles (NPs) on human health as well as the environment. Such knowledge is of great importance for nanotechnology to grow in a responsible and sustainable manner.

The most common definition of nanoparticles includes particles ranging in size from 1 to 100 nm in diameter in at least one dimension under the term (Oberdorster et al., 2005; SCENIHR, 2009). Due to their unique size, they tend to posses novel physical, chemical and biological properties that make these materials superior. Silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2-NPs), which are among the top five NPs in pharmaceutical products, building materials and other consumer products, are pivotal in the development of nanotechnology (Cho et al., 2009; Park et al., 2010; Shukla et al., 2011).

While there are various potential routes of exposure to NPs, inhalation and ingestion appear to be the main routes. Some studies have reported that NPs are able to cross the blood–testis and blood–brain barrier (De Jong et al., 2008; Lankveld et al., 2010). Of particular concern is the fact that AgNPs have been documented to cross the blood–testis and blood–brain barrier in mice (Lankveld et al., 2010) and rats (Gromadzka-Ostrowska et al., personal communication).

NPs are capable of binding to cells as well as macromolecules like proteins and DNA (AshaRani et al., 2009b). When NPs come in contact with cells they are taken up by a variety of mechanisms that can lead to activation of cellular signalling processes producing reactive oxygen species (ROS), inflammation and finally cell cycle arrest or cell death (Ahamed et al., 2008, 2010; AshaRani et al., 2009b). Reduced rate of proliferation, impaired mitochondria function and induction of apoptosis and/or necrosis are among changes which have been observed in AgNP-exposed cells (Schrand et al., 2008; AshaRani et al., 2009a). Toxicity of AgNPs in higher eukaryotes have also been reviewed (Kruszewski et al., 2011). Notably, AgNPs are capable of entering the nucleus, and as such directly or indirectly interacting with nuclear material (AshaRani et al., 2009b; Kruszewski et al., 2011), leading to alterations in DNA integrity or affecting its synthesis. These perturbations may – via the causation of DNA damage or inhibition of cellular processes – result in the formation of mutant or tumorigenic cells. When such processes concern germline cells, the result may be altered spermatogenesis and fertility, subsequently affecting the reproduction rate and health of the offspring (Ema et al., 2010).

Reproductive toxicants targeting the germ line have the potential to cause alterations that can be passed on by genetic or epigenetic mechanisms to the next generation. Although there is

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doi:10.1016/j.tox.2011.10.022

increasing concern for the potential effects of the use of NPs on reproductive health, only few studies have been performed on testicular cells. The present study is therefore aimed at evaluating the cytotoxicity and DNA-damaging potential of AgNPs in NT2 cells and primary mouse testicular cells, employing TiO_2-NPs as a benchmark. We show that AgNPs are more cytostatic and cytotoxic in testicular cells compared to TiO_2-NPs, and tend to be more genotoxic.

2. Materials and methods

2.1. NPs and their characterization

AgNPs were purchased from Plasmachem GmbH, Germany. TiO_2-NPs were obtained from the European Commission Joint Research Center. The nominal sizes of AgNPs obtained were 20 and 200 nm, whereas TiO_2-NPs were 21 nm (referred here to as Ag20, Ag200 and TiO_2-NP, respectively). NPs were dispersed in dH2O/10X BSA/10 PBS in 8:1:1 ratio of 2 mg ml^{-1} stocks, as previously described (Bihari et al., 2008). The hydrodynamic sizes of NPs after the dispersion procedure were determined by dynamic light scattering (DLS) measurements using a Zeta-sizer S (Malvern Instruments, Malvern, United Kingdom). The mean diameters of the Ag20, Ag200 and TiO_2-NPs were 154.6, 266.2 and 259.0 nm, respectively as judged by DLS results. Of note, NPs were freshly prepared before each exposure as they tend to agglomerate in suspension (Bihari et al., 2008).

2.2. Chemicals and culture media

Trypsin, Trypan-blue, EDTA, Tris-base, HCl, Triton-X, Hepes, KCl, BSA, KOH, NaH_2PO_4 and lactic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). NaOH, NaCl, DMSO were purchased from VWR (Radnor, PA, USA). DMEM medium with 25 mM Hepes, 4.5 g l^{-1} glucose, without L-glutamine and RPMI medium with 25 mM Hepes, L-glutamine were purchased from Medprobe (MN, USA). All other chemicals were purchased from commercial sources and were of analytical grade.

2.3. Animals, cell cultures and treatments

Animals used in this study were 8 to 12 week old male 8-oxoguanine DNA glycosylase knockout mice (Ogg1$^{-/-}$ KO), defective in the repair of oxidated purine bases. These mice derived from the C57BL/6 mice and 129SV (Klungland et al., 1999), crossed with Big Blue® C57BL/6 mice (Stratagene, La Jolla, CA, USA) to achieve Ogg1$^{-/-}$ Big Blue® mice, obtained as a generous gift from Klungland and coworkers, were bred in-house. Isogenic Ogg1$^{+/+}$ (WT) and Ogg1$^{-/-}$ strains in similar background (Big Blue® C57BL/6) were generated after back-crossing the Ogg1$^{-/-}$ mice with C57BL/6 mice (Stratagene, La Jolla, CA, USA) (Olsen A.K., personal communication). The mice were housed in plastic cages at room temperature of 20–24 °C, relative humidity of 55 ± 5% and 12 h light/dark cycle. Mice were fed with standard chow and drinking water was provided *ad libitum*. WT and mOgg1$^{-/-}$ KO mice were used and were sacrificed by cervical dislocation; testes were pooled from a minimum of two mice each time, in order to minimize effects from individual variations. The Evenson clip was employed in the preparation of testicular cells (Evenson et al., 1989). The resulting primary cell population comprised (approximate relative proportions obtained from historical controls), 85% haploid cells (spermatids), 5% tetraploid cells (spermatocytes and dividing cells) and 10% diploid cells (somatic cells, spermatogonia and secondary spermatocytes) (Wiger et al., 1995). Cells were maintained in RPMI medium with 25 mM Hepes, L-glutamine and supplemented with 10% FCS, 1% of penicillin and streptomycin (P/S), lactate (5 mM) and pyruvate (0.1 mg ml^{-1}) in 5% CO2, 5% O2 at 32 °C. The experiments were performed in conformity with the laws and regulations for animal experiments in Norway and were approved by the local officer of the Animal Board under the Ministry of Agriculture in Norway.

The pluripotent human testicular embryonal carcinoma, NTERA-2 cl.D1 cell line (NT2) purchased from the American Type Culture Collection was maintained in DMEM medium with 25 mM Hepes, 4.5 g l^{-1} glucose, without L-glutamine and supplemented with 10% FCS, 1% P/S and kept in 5% CO2 at 37 °C. Cells were routinely maintained in logarithmic growth phase at 1–9 × 10^6 cells/75 cm^2 flasks by splitting twice a week. Cells were seeded near confluence a day before treatment and medium was refreshed before exposures. Appropriate controls containing vehicle were included in each experiment.

2.4. Determination of cell metabolic activity by the MTT assay

Cells were exposed to NPs (10/50/100 μg ml^{-1}, equivalent to 7.8/15.6/31 μg cm^{-2}, respectively) for 24/48/72 h in 96 well plates. To each well 100 μl of 3 mg ml^{-1} 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in PBS was added followed by incubation for 4 h at 37 °C. For NT2 cells, media was carefully removed from the wells after the incubation period, whereas plates containing suspension of the primary testicular cells were centrifuged 250 × *g* for 5–10 min before removal of media. DMSO (100 μl) was then added to each well and the plate was shaked on a plate shaker for 15 min to obtain dye dissolution. The absorbance was measured at 570 nm by a TECAN Sunrise plate reader.

2.5. Cell death analyses by PI/Hoechst staining

To directly assess the cell death after exposure to the NPs, floating and attached cells (0.5 × 10^6 cells) were stained with propidium iodide (PI; 10 μg ml^{-1}) and Hoechst 33342 (5 μg ml^{-1}), and plasma membrane integrity and nuclear morphology were evaluated under the microscope. After 30 min incubation cells were centrifuged, resuspended in FCS and smears on microscopic glass-slides were prepared and quickly air dried. Cell morphology was evaluated using a Nikon Eclipse E 400 fluorescence microscope (magnification 400/1000×). Cells with clearly or partially condensed chromatin and/or fragmented nuclei were counted as apoptotic cells. All the PI-stained cells exhibiting a homogeneously stained nucleus with rounded morphology were counted as necrotic cells. Non-apoptotic cells, excluding PI, were categorized as viable cells.

2.6. Single cell gel electrophoresis (comet assay)

At different time points after exposure to NPs, media were removed and attached cells (NT2) were trypsinized and resuspended at 1 million cells/ml in medium with 10% FCS. Notably, the primary testicular cells did not require trypsinization. Samples were analysed for DNA damage using the comet assay, carried out as previously described (Duale et al., 2010; Hansen et al., 2010) with minor modifications. Briefly, cells suspended in 0.68% LMP agarose were moulded as 4/8 μl droplets/gel volume) onto 96/48-gel format GelBond films, respectively (Gutzkow et al., personal communication). Notably, these films were attached to plastic frames to facilitate subsequent treatment steps. After lysis, films were treated with or without a bacterial Fpg enzymic extract as previously described (Boiteux et al., 1990) with modifications (Olsen et al., 2003; Duale et al., 2010). After electrophoresis (0.8 V/cm, 20 min, pH 13.2) and neutralisation, films were fixed in ethanol and dried. Rehydrated samples were stained with SybrGold (0.15 μl/ml in TE-buffer pH 7.4, 20 min) and scored with Perceptives Comet IV (Perceptive Instruments Ltd., Suffolk, UK) for the detection of DNA strand breaks and alkali-labile sites. DNA breakage was expressed as a percent of the intensity of DNA in the comet tail. Nuclei extracts from NT2 and mice primary testicular cells exposed to 0–5 Grays of X-rays were employed as positive controls as these exhibited linear increments in DNA tail intensities for up to 15 Grays.

2.7. Cytokine asssay

Supernatants from NP-exposed cells were removed and analysed by the enzyme-linked immunosorbent assay (ELISA) for the release of IL-8/MIP-2, IL-6, and TNF-α according to the manufacturer's instructions. The TECAN Sunrise plate reader with the Magellan V 1.10 complete software was employed for absorbance readings.

2.8. Statistical analyses

One way ANOVA employing the Holm–Sidak method was used routinely, and $P < 0.05$ was defined as a statistically significant change.

3. Results

3.1. Cytotoxic effects of NPs inNT2 and primary mouse testicular cells

Fig. 1 shows micrographs of NT2 cells exposed to 100 μg ml^{-1} NPs for 24 h, with clearly reduced number of cells for the nano and submicron particles (Ag20 and Ag200), compared to control (untreated). The cellular metabolic activity, reflecting somehow the rate of proliferation of testicular cells, as assessed by the MTT assay, was reduced with increased exposure time and concentrations of Ag20 and Ag200 (Fig. 2). The data indicate that Ag20 and Ag200 affected the mouse primary testicular cells, both WT and KO (Ogg1$^{-/-}$) at very low concentrations (10 μg ml^{-1}), whereas the human testicular carcinoma cell line NT2 seemed to be less prone to the toxic effects of AgNPs. This was most pronounced at 48 h (Fig. 2D–F). No marked differences in toxicity response were observed between WT and KO testicular cells. At 24 h and at maximum (100 μg ml^{-1}) AgNP concentration, the metabolic activity of all three cell types was reduced to approximately 50% of controls. Prolonged exposures for 48 h revealed that the effect of Ag20 (100 μg ml^{-1}) on the metabolic activity was persistent, with

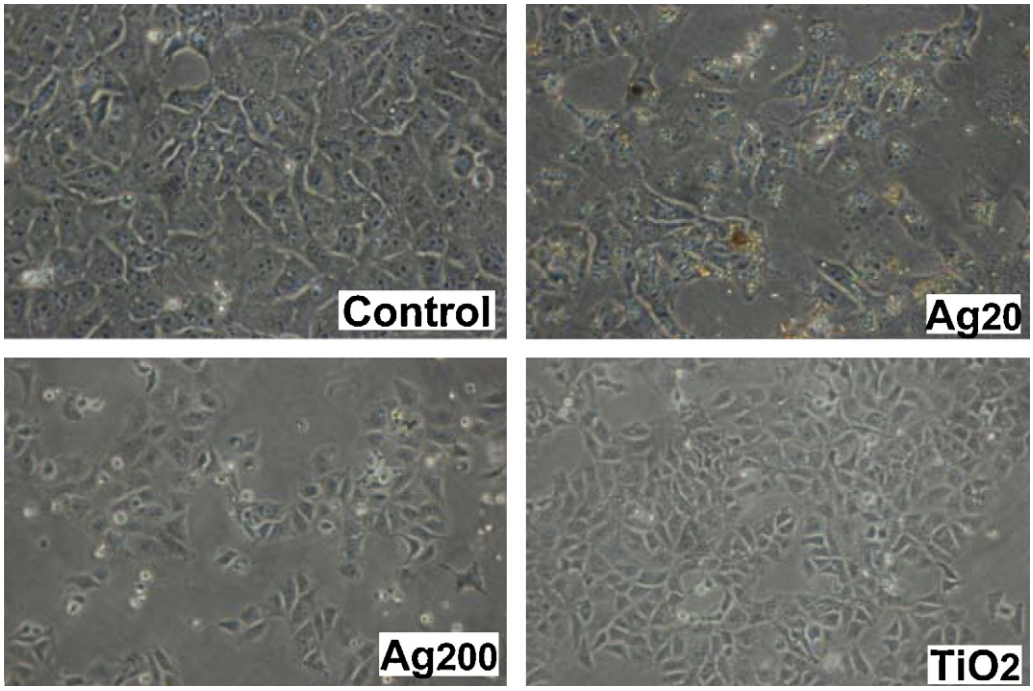

Fig. 1. Effect of Ag20, Ag200, and TiO_2-NPs on cell numbers. NT2 cells were exposed to 100 μg ml^{-1} NP concentrations for 24 h and pictures were taken with a light microscope at 200× magnification.

average reduction to 22.5% and 27.5% in WT and KO cells, respectively, compared to controls (Fig. 2 D–F). In the NT2 cells, the toxic effects of Ag20 and Ag200 seemed less pronounced, and no difference between the nano-sized and submicron silver particle was observed at the lower concentrations. At 100 μg ml^{-1} (24 and 48 h), however, the data suggest a reduction in cellular metabolic activity, and this change was significant for Ag20 after 48 h exposure (Fig. 2F).

With respect to TiO_2-NPs, no sign of reductions in cell number/proliferation was observed. In contrast, TiO_2-NPs appeared to elicit an increase in the rate of proliferation in WT and KO primary mouse cells (Fig. 2A, B, D, and E). This increase ranged from 140% at 24 h to 180% at 48 h and was observed at all concentrations of TiO_2-NPs used.

PI/Hoechst staining was performed to determine cell death caused by Ag20, Ag200 and TiO_2-NPs, and to confirm the results obtained from the MTT assay. We observed similar concentration-dependent cytotoxic effects of Ag20 and Ag200 on NT2 cells as with the MTT assay (Fig. 3). Fig. 3A shows decreasing numbers of viable cells with increasing concentration of AgNPs. Statistically significant increase in per cent necrotic cells was observed at the lowest concentration of 12.5 μg ml^{-1} for both Ag20 and Ag200. Both AgNPs markedly induced cell necrosis, with 23% and 15% necrotic cells at 100 μg ml^{-1}, respectively, compared with 5% in controls (Fig. 3B). TiO_2-NPs did not induce any significant increase in necrosis, although a slight trend was observed (Fig. 3).

With regards to the primary mouse testicular cells (WT or KO), a simplified approach based solely on PI exclusion was employed, since it was challenging to differentiate between viable and apoptotic cells in this model. This is because nuclear DNA material in normal sperm cells – being one constituent of the spermatogenic cell suspension – is tightly packed, and these cells may fluoresce even when apparently undamaged as in controls [when viable]. PI-negative cells were considered viable and PI-positive cells (necrotic) determined as a fraction of total number of cells, We observed a general average of 30–50% necrotic cells in particle-exposed primary testicular cell samples as compared to 15% in controls, with no apparent increase with increasing concentrations (data not shown). Notably, necrotic cell death is usually accompanied by inflammation. However, cytokine release at different NP concentrations and time points did not show any increase from control levels and the levels were below detection in some cases in both NT2 and primary mouse cells (data not shown).

AgNPs have been shown to induce apoptosis in other cell types (Kalishwaralal et al., 2009; AshaRani et al., 2009b). Our data reveal that apoptosis is elicited in Ag200-exposed NT2 cells with a significant effect at the highest concentration (100 μg ml^{-1}) of Ag200 (Fig. 3C). A trend was observed also for Ag20, but was not significant even at the highest concentration. Upon TiO_2-NPs exposure, negligible effects were observed (Fig. 3).

3.2. DNA damaging effects of NP-exposed NT2 and primary mouse testicular cells

Exposed cells were analysed with the comet assay to measure induction of DNA-strand breaks by AgNPs. Oxidative purine base damage was analysed by treating parallel samples of lysed cells, embedded in agarose, with the formamidopyrimidine-DNA glycosylase (Fpg) enzyme followed by standard alkaline electrophoresis. Fpg is an enzyme of the base excision DNA repair pathway, and recognizes and removes mainly 7,8-dihydro-8-oxoguanine (8-oxoguanine) (Boiteux et al., 1990; Olsen et al., 2003; Duale et al., 2010). Generally, our results showed low levels of NP-induced DNA strand breaks in NT2 cells or the primary testicular cells, although a positive concentration-dependent trend was observed in NT2 cells in the comet assay without Fpg (Fig. 4A). Ag200 seemed to cause the highest level of damage, with about 25% DNA intensity in tail at the100 μg ml^{-1} concentration, whereas Ag20 and TiO_2-NP caused approximately 15% and 10% strand breakage, respectively, at the same concentration in NT2 cells. In contrast, the NPs seemed to cause little to no DNA-strand breaks in testicular cells derived from either WT (Fig. 4B) or KO (Ogg1$^{-/-}$) (Fig. 4C) mice. Additionally, no

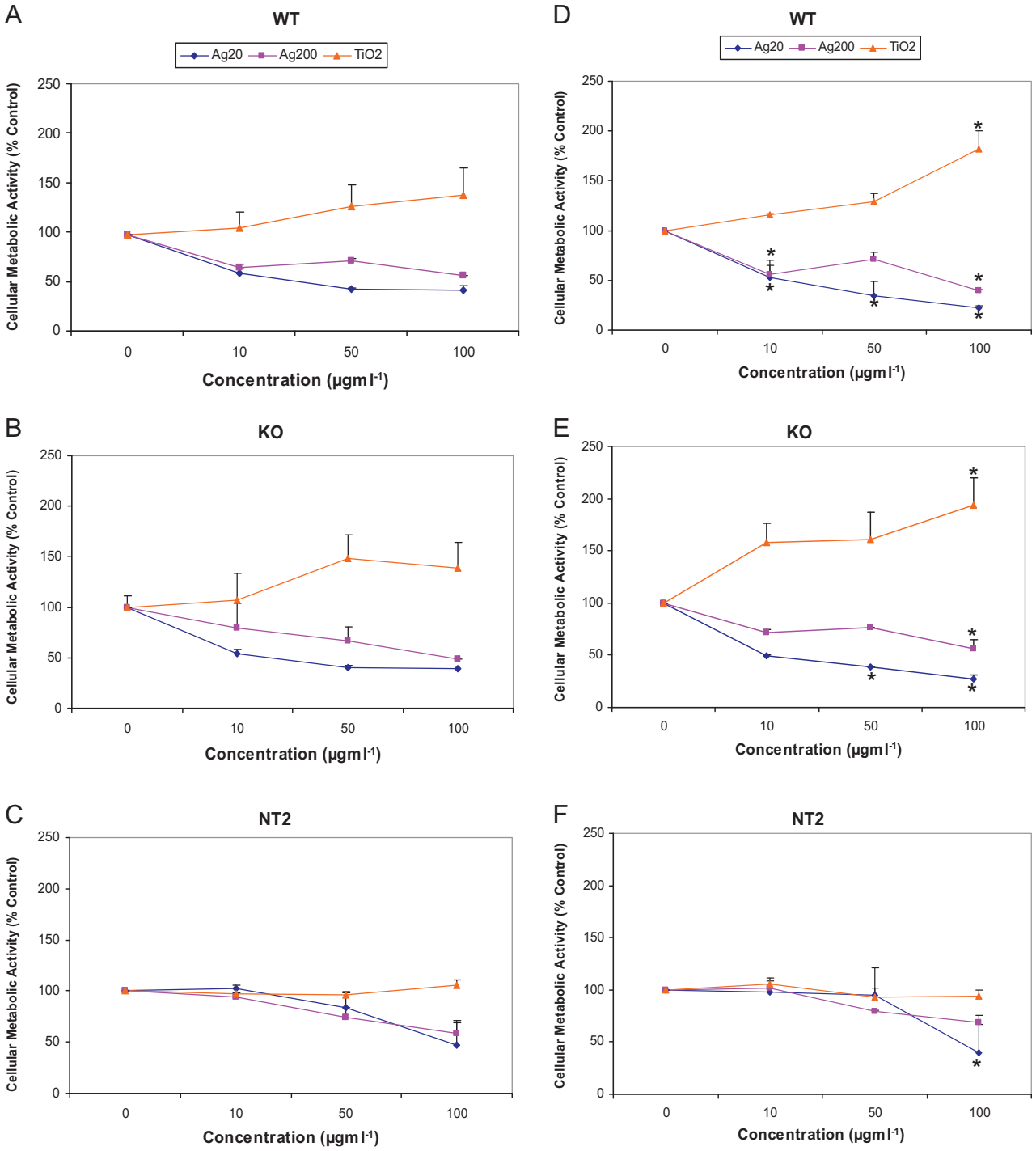

Fig. 2. Effect of Ag20, Ag200, and TiO_2-NPs on cellular metabolic activity. NT2 and primary testicular cells were exposed to the indicated NP concentrations for 24 h (A–C) and 48 h (D–F), stained with MTT dye and absorbance readings measured were expressed as % of control. Data are presented as means ± S.E. of at least 2 separate experiments each with a minimum of 6 parallels. Asteriks (*) indicates statistical significance between controls and treated, $P < 0.05$.

significant increase in oxidative DNA damage was observed in any of these experiments.

4. Discussion

Several particle features, such as type, size, zeta potential, dispersion/agglomeration status, as well as potential interaction with biomolecules, influence NP toxicity and hence their effects in humans. Generally, size (and hence the surface/mass ratio) has been considered as the most important factor for the toxicity of NPs (Hubbs et al., 2011). In the present study, we attempted to minimize variations due to inherent physical/biological properties of the particles used. As stated in the materials and method section, NPs suspensions were freshly prepared for each exposure, and a concentrated BSA solution was added immediately after sonication in water since agglomeration and/or aggregation tend to increase with time in suspension (Bihari et al., 2008). Uncontrolled agglomeration may heavily affect the particle size distribution. Some studies also report that particle uptake is dependent on competition between the thermodynamic driving force needed for membrane

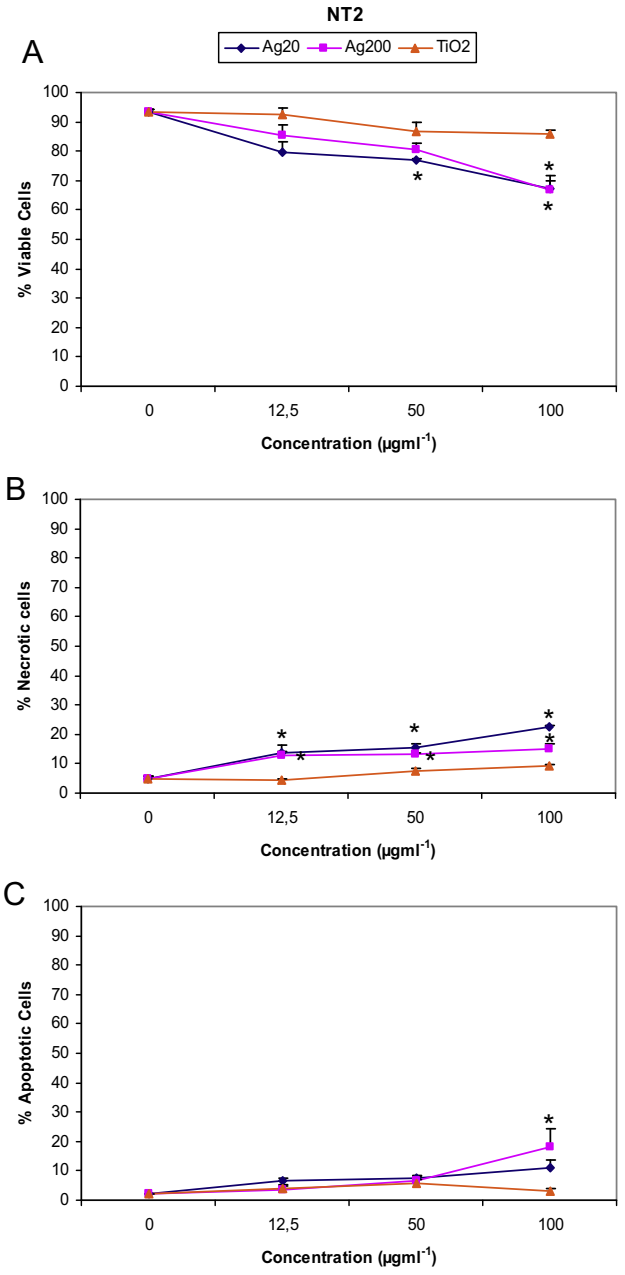

Fig. 3. NP-induced cell death in testicular cells. After exposure to the indicated NP concentrations for 24 h, cells were stained with PI and Hoechst 33342 and analysed by fluorescence microscopy as described in Section 2. Microscopic counts of NT2 cells showing, viable (A), necrotic (B) and apoptotic (C) cells after exposure to Ag20, Ag200 and TiO_2-NP. Data are presented as means ± S.E. of at least 3 separate experiments. Asteriks (*) indicates statistical significance between controls and treated, $P < 0.05$.

wrapping and receptor diffusion kinetics (Gao et al., 2005; Chithrani et al., 2006; Chithrani and Chan, 2007; Zhang et al., 2009). According to this hypothesis, for small-sized particles an unfavourable increase in free energy results in a decrease in the degree of cellular uptake (Gao et al., 2005; Chithrani et al., 2006; Chithrani and Chan, 2007; Zhang et al., 2009). Thus small particle size does not necessarily lead to better uptake and hence increased toxicity (Chithrani and Chan, 2007). Additionally, it has been reported that the surface chemistry of NPs influence interparticle interactions, hence particle distribution and in effect transport across membranes (Stobiecka and Hepel, 2011) and genotoxicity (Ahamed et al., 2008). Ahamed et al. (2008) showed that polysaccharide-coated AgNPs caused more severe DNA damage than uncoated AgNPs in mouse embryonic stem cells and fibroblasts.

Testicular toxicity is pivotal in assessing reproductive toxicants since the former may cause testicular dysfunction or spermatogenic disruption, resulting in male reproductive health-related problems such as infertility and/or poor sperm quality that could potentially result in birth defects. In our models, NPs caused differential toxic effects in the testicular primary cell populations. The MTT assay reflects mitochondria function, and in particular mitochondrial red-ox metabolism in live cells (Monteiro-Riviere et al., 2009). Our results are thus in accordance with a proposed mechanism of action for AgNPs by AshaRani et al. (2009b), involving disruption of the mitochondrial respiratory chain and in effect interruption of ATP synthesis. In contrast, TiO_2-NPs seemed to augment mitochondrial activity in testicular cells from both WT and KO ($Ogg1^{-/-}$) (Fig. 2). However, this activation was only significant at 48 h, with 182% and 194% in WT and KO cells, respectively at 100 μg ml^{-1} NP concentration, although evident at 24 h (Fig. 2). Although this response should be carefully interpreted, it may indicate that TiO_2-NPs may possess the capacity to elicit a release of cell growth factors above normal levels, or switching off the programmed cell death (PCD) machinery by induction of anti-PCD factors such as the Bcl-2 family of proteins. Such a property may promote the formation of mutant cells and set a stage for the onset of tumorigenesis, but this needs to be verified. Interestingly, TiO_2-NPs have been reported to cause lung cancer in rodents (Borm et al., 2004; Dankovic et al., 2007).

Using PI/Hoechst staining to assess necrotic/apoptotic responses revealed a similar pattern for necrosis as observed for the MTT responses. With respect to apoptosis, our data (Fig. 3) revealed that Ag200 – per unit mass in suspension – caused more apoptotic cell death at the highest concentration in the human NT2 cells compared with Ag20, which appeared to induce more necrotic cell death at all concentrations. Different mechanisms may be at play possibly due to different modes of cellular uptake of the nano- and micron-sized AgNPs. Notably, the primary mouse testicular cells seemed more sensitive to the particles compared to the NT2-cell line, at relatively low particle concentrations (Fig. 2). No significant difference was observed in the response of the WT versus KO cells, when incubated with NPs *in vitro* (data not shown). TiO_2-NPs caused the least cell death, in NT2 cells (necrosis/apoptosis) and primary testicular cells (necrosis; data not shown). Notably, there are other reports of apoptosis and/or necrosis in different cell types exposed to AgNPs (Arora et al., 2008; Kalishwaralal et al., 2009; AshaRani et al., 2009b; Gopinath et al., 2010), but findings with regards to the cytotoxic effects of TiO_2-NPs in other models are contrasting (Kang et al., 2008, 2011; Shukla et al., 2011; Zhang et al., 2011).

Many engineered NPs, including AgNPs have been found to cause genotoxic effects, such as DNA-strand breaks, point mutations, and oxidative DNA adducts (Ahamed et al., 2008, 2010; Kim et al., 2009; Choi et al., 2010; Foldbjerg et al., 2011). NPs easily cross the nuclear membrane and they can therefore interact with DNA directly or indirectly although the exact mechanism for this interaction is not yet known. Some of these alterations and/or damage to DNA, when occurring in spermatozoa may cause spermatogenic defects that could eventually result in trans-generational defects should the genomic stability of sperm cells be compromised (Lucas et al., 2009). Although mechanistic investigations are needed in this area, there are indeed reports indicating reproductive and developmental impairment of organisms exposed to NPs (Yoshida et al., 2009, 2010; Ema et al., 2010). AshaRani et al. (2009b) reported a dose-dependent damage to DNA after AgNP treatment in human lung fibroblasts, IMR-90 and glioblastoma cells, U251. However, Martinez-Gutierrez et al. (2010) observed no such effect in AgNP-exposed THP-1 monocytes. We observed a

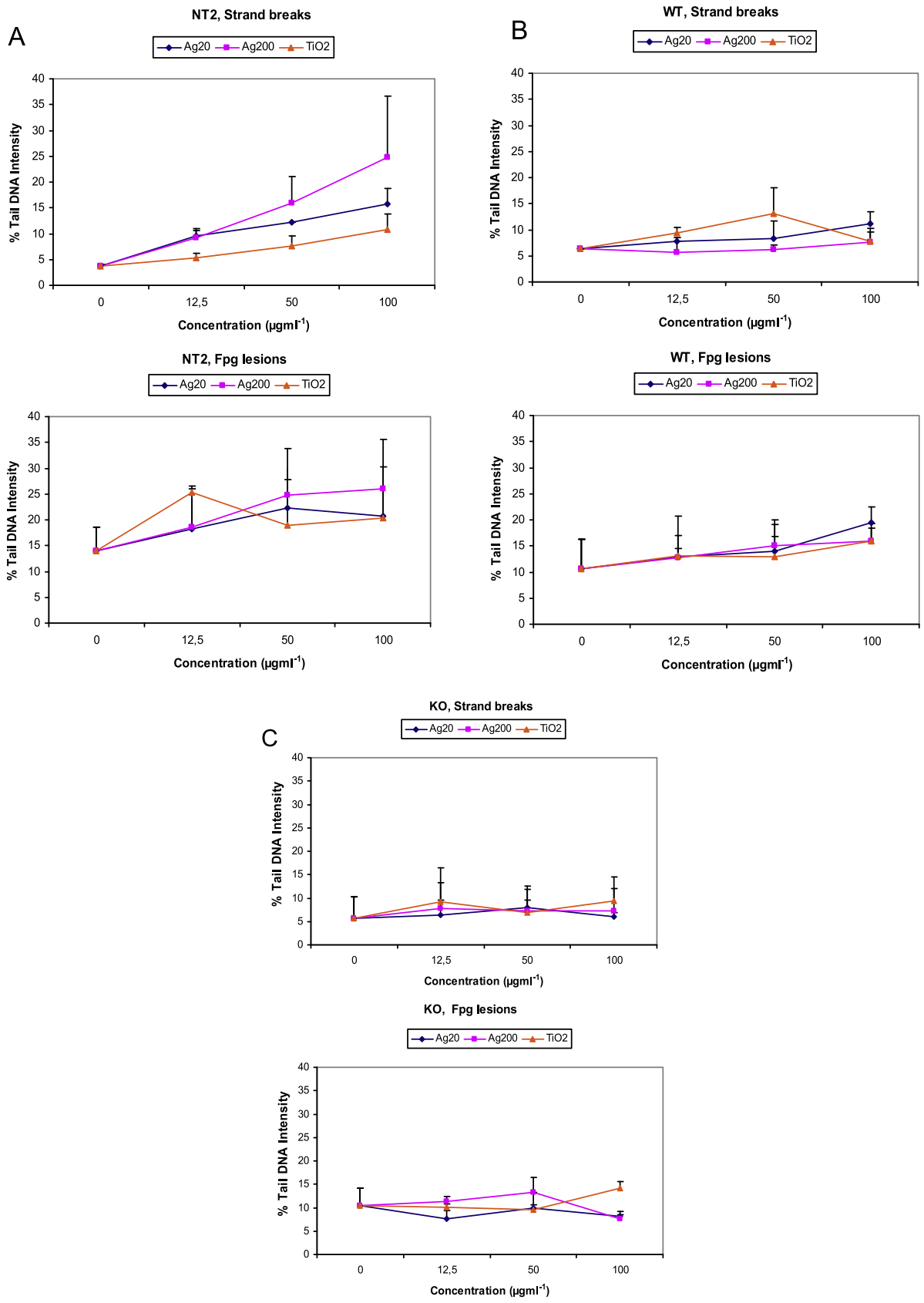

Fig. 4. Effect of Ag20, Ag200, TiO_2-NPs on DNA-damage in testicular cells. Cells were exposed to 12.5, 50 and100 µg ml^{-1} NP concentrations for 24 h. DNA-strand breaks and alkali-labile sites were detected by single cell gel elctrophoresis in alkali conditions, with or without FPG enzyme and scored employing "Perceptives Comet IV". (A) NT2 cells, (B) wild type primary testicular cells, and (C) KO primary testicular cells with strand breaks, upper panels and Fpg lesions, lower panels. Data are presented as means ± S.E. of at least 2 separate experiments.

concentration-dependent increase in DNA-strands breaks especially in NT2-cells, although not statistically significant (Fig. 4). Of interest, TiO_2-NPs caused a considerable level of DNA damage compared with controls, however, low.

ROS formation was suggested to be a key event in DNA damage induction in A549 cells, a human lung cancer cell line, exposed to AgNPs (Foldbjerg et al., 2011). Similar findings have also been reported in other human cell lines by AshaRani et al. (2009b). Arora et al. (2008) demonstrated clear signs of oxidative stress as measured by decreased GSH/SOD and increased lipid peroxidation in AgNP-exposed human fibrocarcinoma HT-1080 and human skin carcinoma A431 cells, which may have led to the observed DNA fragmentation. TiO_2-NPs have also been reported to cause oxidative damage (Kang et al., 2008; Shukla et al., 2011). Based on these reports, we investigated if selected NPs induced oxidative-base DNA damage in testicular cells by incorporating the Fpg enzyme into the Comet assay. The formation of 8-oxo-7,8-dihydro-2'-guanosine (8-oxoG), representing an important mutagenic base damage, is reported to be a relevant indicator of oxidative base DNA damage that may lead to DNA dysregulation (Shibutani et al., 1991). Notably, during studies of repair of oxidative DNA lesions in spermatogenic cells from rodents and human testicular biopsies it was observed that testicular cells from humans exhibited considerably less efficient repair of oxidative damage compared to rodent testicular cells (Olsen et al., 2001, 2003). Due to specific repair deficiencies in the human male germ cells, we have hypothesized that humans are more susceptible to oxidative agents compared to rodents. We therefore used the $Ogg1^{-/-}$ mice with defective repair of oxidative DNA damage, as a model for human male germ cells, allowing a direct comparison of the importance for NP genotoxicity of an important pathway for the repair of oxidative lesions. The model allows accumulation of oxidative DNA lesions enhancing the sensitivity of endpoint assays and gives rise to sperm with levels of oxidative DNA damage similar to that of human sperm (Brunborg et al., 2007). Our results show that there is some tendency of increased oxidative damage especially in NT2 cells, however, low as judged by the extra lesions detected when employing Fpg in the Comet assay (Fig. 4). Our findings indicate that ROS formation, leading to oxidative purine base DNA damage may not be the main mechanism of NPs action in our models.

Some studies have reported that NPs cause inflammation, including increase in the release of pro-inflammatory cytokines, such as IL-8 and TNF-α (Hubbs et al., 2011; Inoue and Takano, 2011). The selected NPs in our studies did not affect the release of cytokines in neither NT2 nor primary cells (data not shown). This was unexpected, as necrosis is usually accompanied by the release of inflammatory mediators and in addition, testicular cells are known to possess chemokine receptors (Hutson, 1993; Kern et al., 1995; O'Bryan et al., 2005). Furthermore, Sandberg W et al. (unpublished data) obtained marked release of IL-8 and TNF-α in adenocarcinomic human alveolar basal epithelial, THP1 as well as human bronchial epithelium, Beas2B cells, with the same NPs used in this study and same dispersion routine. These findings make it less likely that the lack of cytokine release observed in our experiments result from a bias in the system. It is possible that the type of necrotic cell death in our system may not be eliciting cytokine release, in which case it may be programmed cell death (Matsumura et al., 2000; Asare et al., 2009a,b). However, this observation could also be explained by a shift in the apoptotic process into necrosis due to lack of energy needed to complete apoptosis.

5. Conclusions

Taken together, AgNPs appear to be more cytotoxic as well as cytostatic compared to TiO_2-NPs in the primary testicular cells and cell line examined. With regards to genotoxicity, although weak and cell-type-specific, both NP types showed a tendency to cause DNA damage. Considering that TiO_2-NPs appeared to activate the rate of cell proliferation, both NP types may have possible implications on reproduction as well as human and environmental health in general. Additionally, this study adds to existing knowledge that NPs type, and not solely size may be the limiting factor in their exerted toxicity. Moreover, cell-type-specific responses seem to be important. Further studies in in vivo models will shed more light on the impact of these NPs on human and environmental health.

Conflict of interest

None.

Acknowledgements

This study was funded by the Polish-Norwegian Research Fund, Contract no. PNRF-122-AI-1/07. The support of other PNRF-122 team members not listed herein is greatly appreciated. Thanks to Daniel Pharm for technical assistance with the Comet assay.

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