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# Treatment with silver nanoparticles delays repair of X-ray induced DNA damage in HepG2 cells

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**Abstract.** Nanoparticles (NPs) defined as particles having at least one dimension below 100 nm have been applied in the last decade in industry and medicine. Recently, there is an increased concern about the biohazard aspect of the presence of NP in consumer goods and in the environment. Silver NP (Ag NP) cause oxidative stress in mammalian cells in result of generation of reactive oxygen species (ROS). This results in genotoxicity and mutagenicity, disturbed mitochondrial respiration, slowed proliferation and cell death. Using the alkaline comet assay, we examined the effect of combined treatment with Ag NP 20 nm or 200 nm and X-rays (2 Gy) in HepG2 cells. In addition, combined treatment with X-rays and titanium dioxide NP ($TiO_2$ NP) 21 nm was also studied. No effect of NP pre-treatment on X-ray induced initial deoxyribonucleic acid (DNA) damage levels was observed for all three NP. In contrast, Ag NP treatment preceding exposure to X-rays caused a marked decrease in the rate of single strand break rejoining. The effect was particularly strong for Ag NP 20 nm. $TiO_2$ NP pre-treatment had no effect on DNA repair.

**Key words:** silver nanoparticles (Ag NP) • titanium dioxide nanoparticles ($TiO_2$ NP) • DNA break rejoining • alkaline comet assay • ionizing radiation • combined treatment with nanoparticles + X-rays

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## Introduction

The rapid development of nanomaterials during the last decade brought a technological breakthrough in many fields of human activity. NPs defined as particles having one or more dimensions of the order of 100 nm or less have found many applications in industry and medicine. The last few years brought, however, an increased concern about the biohazard aspect of the presence of NP in consumer goods and in the environment [5, 17, 33, 36]. The variety of nanomaterials, their size, shape and coating give them different biological properties and makes biohazard estimation very complicated [17, 25, 39]. In addition, the biological effect of NP likely depends on the target tissue and the way of entry. Further complications are brought about by possible interactions with other biologically active agents. Hence, a detailed study is necessary for each NP type and cell or tissue type, as well as interacting chemical or physical agents.

So far, Ag NP which have antibacterial properties have been integrated into hundreds of consumer products, including cosmetics, odor-reducing clothing and underwear. Although Ag NP belong to the most often studied ones, the mechanisms of their biological effects are still not fully understood. There is a general agreement that the various, NP-induced end effects at the level, including genotoxicity and mutagenicity, disturbed mitochondrial respiration, slowed prolifera-

tion and apoptotic death may have a common cause, which is the oxidative stress [3, 30]. It is also clear that Ag ions are not responsible for the NP effects or are responsible to a very small degree [20].

As mentioned above, NP may interact with other biologically active agents. So far, not many studies have been performed concerning this problem. Applications of ionizing radiation in nanotechnology are mostly focused on NP synthesis, nanostructure modification or functionalization. Interaction of NP with ionizing radiation to our best knowledge has been examined so far only in the context of enhancement of radiation effects by gold NP and their potential applications in medicine [11, 41] or the use of NP in bioimaging [18, 26]. Therefore, we undertook experiments aimed at determination of the possible interaction of Ag NP or $TiO_2$ NP and X-rays in a cellular *in vitro* system with DNA damage induction and repair as end-points.

## Materials and methods

### Nanoparticle preparation

Ag NP of nominal size 20 and 200 nm were purchased from Plasmachem GmbH, Germany. $TiO_2$ NP powder of nominal size 21 nm anatase/rutile polymorphs was purchased from Degussa-Evonik (Essen, Germany) and kindly obtained from the European Commission Joint Research Center (EC JRS) depository. NP stock solutions were prepared at a concentration of 2 mg NP in distilled water with sonification at 420 J/cm$^3$; 100 µl of bovine serum albumin and 100 µl of a 10× concentrated phosphate buffered saline were given immediately to 800 µl aliquots of suspension. Since NP tend to aggregate in biological media, the sizes of NP aggregates in suspension were determined by the dynamic light scattering (DLS) method (Zatasier S, Malvern Instruments, Malvern, United Kingdom). Mean diameters of the Ag 20 nm, Ag 200 nm and $TiO_2$ 21 nm NPs were 130.6, 266.1 and 126.3 nm, respectively. The hydrodynamic radius or diameter as measured by the DLS method often is different from the nominal diameter as supplied by the producer. In the described case, the possible reason of discrepancy is polydispersion that could stem from a wide size distribution of particles present, bimodal populations, aggregation or agglomeration.

### Cell culture and treatment

Human hepatocellular liver carcinoma (HepG2) cells were kept in culture in Williams Medium E (Sigma-Aldrich) supplemented with antibiotics (Antibiotic-Antimycotic, Sigma-Aldrich) and 10% fetal calf serum (Gibco). Appropriate number of cells was seeded on 5 cm Petri dishes and left to settle down without NP for 24 h to obtain optimal cell attachment to the plastic surface. Subsequently, the nanoparticle suspension was added to the cell culture to obtain the final concentration of 100 µg/ml for 2 h. After treatment, the medium containing NP was removed and the plates were washed twice with 5 ml of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4, PBS). After washing, cells were trypsinized and suspended in fresh medium. Immediately afterwards, X-irradiation was carried out in an ice water bath, with the use of a Smart200 (Yxlon) defectoscope operating at 200 kV and 4.5 mA, with 3 mm Al filtration, at a dose rate of 1.14 Gy/min.

### Alkaline comet assay

The comet assay (single cell gel electrophoresis) was performed as described in [35]. Briefly, an aliquot of cell suspension was mixed with an equal volume of 2% low melting point agarose (Type VII, Sigma), put on a microscope slide pre-coated with 0.5% regular agarose (Type I-A, Sigma) and left on ice. After agarose solidification, the slides were immersed in a cold lysing solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris and 1% Triton X-100, pH 10) or left for 30 min in culture media at 37°C to allow damage repair. After 40 min lysis, the slides were placed on a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (1 mM $Na_2EDTA$ (sodium ethylenediamine tetraacetate) and 300 mM NaOH) and allowed to stay in this buffer for 40 min for DNA unwinding. Next, electrophoresis was performed (1.2 V/cm, 30 min, 10°C). After electrophoresis, the slides were washed with 0.4 M Tris, pH 7.5 (3 × 5 min) and stained with DAPI (4',6-diamidino-2--phenylindole), 50 µl (1 µg/ml).

Basically the same test was applied for the measurement of DNA base damage. Incubation of irradiated cells with the formamido-pyrimidine glycosylase (FPG), BioLabs, was carried out as described in Ref. [24]. Briefly, after lysis, the slides were washed 3 × 5 min with the buffer (40 mM Hepes ((4-(2-hydroxyethyl)--1-piperazine ethanesulfonic acid), 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8) at 4°C. Further, 50 µl of FPG solution ($4.8 \times 10^{-2}$ U) in the buffer was placed on each slide, covered with cover glass and incubated for 30 min in a light-protected box at 37°C. Slides were stained with DAPI (1 µg/ml) and analyzed as described above.

Image analysis of data was performed by the Comet Assay IV Image Analysis System (Perceptive Instruments, UK). Seventy five randomly selected comets per slide were analysed. Percent of DNA in comet's tail was chosen in this report as a measure of DNA damage.

### Statistical evaluation

The statistical significance of difference of means was evaluated using Student's t-test. All experiments were done in three independent repeats.

## Results and discussion

The effect of NP on DNA damage induction and repair was investigated in HepG2 cells. The background level of DNA strand breaks was 1.7% DNA present in the comet tail. As shown in Fig. 1, Ag NP applied as single agents (100 µg/ml, 2 h treatment) induce DNA breakage in these cells. The effect depends on the particle

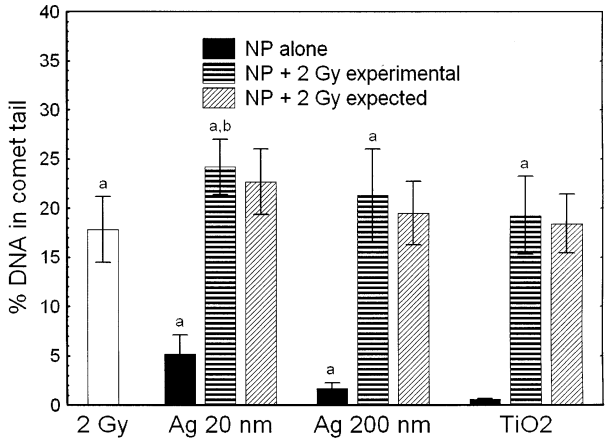


**Fig. 1.** Effect of nanoparticle pre-treatment on induction of DNA damage by X-rays. Value for untreated cells was subtracted. Letter 'a' denotes statistically significant difference vs. untreated, mean ± SD, $n = 4$, $p < 0.05$, letter 'b' denotes statistically significant difference vs. 2 Gy alone, mean ± SD, $n = 4$, $p < 0.05$.

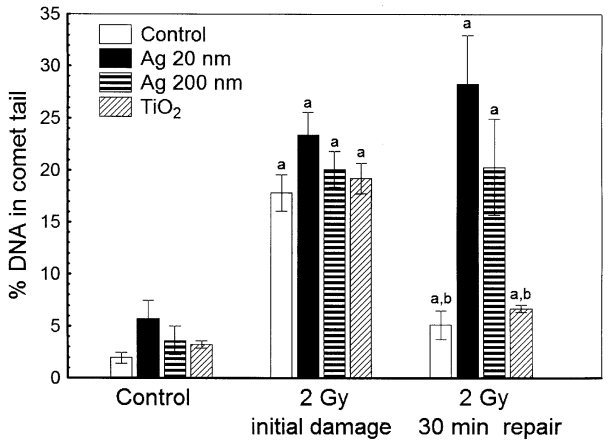


**Fig. 2.** Effect of nanoparticle pre-treatment on repair of X-ray-induced DNA damage. Letter 'a' denotes statistically significant difference vs. unirradiated cells, mean ± SD, $n = 4$, $p < 0.05$; letter 'b' denotes statistically significant difference vs. 2 Gy, mean ± SD, $n = 4$, $p < 0.05$.

size: the smaller NP are more effective than the larger one. This may be due to less effective uptake of 200 nm Ag NP during the 2 h treatment. However, the effect of nanoparticle size and shape on its cyto- and genotoxicity has been reported by several authors [6, 16, 40]. This is likely due to a larger surface to volume ratio in the case of smaller NP, and a more intense generation of ROS on the surface of smaller NP [10]. Interestingly, $TiO_2$ NP caused only negligible DNA damage. When combined with X-irradiation (2 Gy), the damage level in all cases was higher than the damage inflicted by 2 Gy alone. However, only in the case of NP Ag 20 nm this difference was statistically significant. Nevertheless, in all cases the level of damage induced by combined treatment did not differ from that predicted for an additive effect (Fig. 1).

It is commonly accepted that the genotoxic action of Ag NP is most likely due to ROS generation [4, 22, 33]. Hence, the majority of DNA lesions are in fact inflicted by the same molecular mechanism with both treatments, as the indirect effect of X-rays on DNA due to water radiolysis products is high, estimated on 85–90% of damage induced [23]. Thus, it could be expected that combined treatment will give a strictly additive effect and this is indeed the case, as shown in Fig. 1. In our hands, $TiO_2$ NP alone did not induce DNA damage at all; thus, combined treatment resulted in DNA damage similar to that observed for 2 Gy alone.

When NP-untreated cells were irradiated and left for repair, as expected, the level of strand breaks (SB) quickly decreased and reached the level of 29% of the initial damage after 30 min of repair. Similarly, in HepG2 cells treated with $TiO_2$ NP, DNA repair proceeded rapidly, and after 30 min of repair the level of DNA damage averaged 34% of the initial damage (Fig. 2). Apparently, after 30 min, the DNA damage repair process was not yet completed, as the level of remaining DNA breaks was still significantly higher than in control cells. Nevertheless, it seems that repair processes proceeded without major problems. Strikingly, in cells treated with Ag NP, repair of DNA damage was considerably impaired. Actually, Ag NP 20 nm pre-treated and X-irradiated cells show an increase in DNA breakage after 30 min repair (however, this difference is not statistically significant). In cells treated with Ag NP 200 nm, the level of DNA damage after 30 min of repair was similar to that initially induced (Fig. 2).

To our best knowledge, this is the first report on NP effect on X-ray-induced DNA repair, so there are no data available for comparison. We show a clear difference between Ag NP and $TiO_2$ NP. Treatment with the latter NP does not induce DNA damage and this apparently is the key to the marked effect of Ag NP on the repair of X-rays induced DNA damage. Although there is a lack of comprehensive data on the influence of oxidative stress on SB rejoining, as the papers describing post-irradiation events in oxidatively stressed cells are dealing with different end-points (e.g. [12, 19, 29], it might be speculated that slowdown of DNA breakage repair is the effect of the presence of additional oxidatively damaged DNA bases induced by NP. It was proposed by S. Wallace and collaborators [8, 34] that after exposure to ionizing radiation different cellular repair systems compete for the availability of the lesion site (see also review in [32]). They have shown that repair of radiation-induced clustered DNA damage did not occur prior to cleavage of the 8-oxoG by FPG glycosylase, if the opposing single strand break was situated three or six nucleotides away [14]. The same group has also shown that down-regulation of human OGG1 protein, the DNA glycosylase whose main substrate is also 8-oxoG, resulted in reduced radiation cytotoxicity and decreased double strand break (DSB) formation post-irradiation [37]. This supports the idea that the oxidative DNA glycosylases apurinic/apyrimidinic (AP) lyases convert radiation-induced clustered DNA lesions into DNA breaks, including lethal DSB. Indeed, pre-treatment of HepG2 cells with Ag NP resulted in formation of DNA base damage, as revealed by the comet assay modified to recognize damaged bases (Fig. 3). These additional lesions present in DNA may result in slowing down or blocking the repair processes, or in forming of new breaks resulting from their repair [38]. In contrast, in cells pre-treated with $TiO_2$ NP, that did not induce base damage, repair of X-irradiation induced damage was similar to that in cells not treated with NPs.

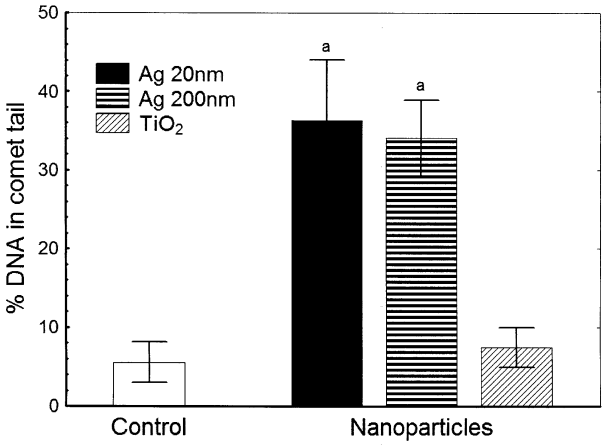


**Fig. 3.** Nanoparticle induced oxidative DNA damage expressed as FPG-sensitive damaged sites. Letter 'a' denotes statistically significant difference vs. untreated cells, mean ± SD, $n = 4$, $p < 0.05$.

As this paper is a preliminary report, no further attempt has been made on explanation of the mechanism of the effect of Ag NP on single strand break (SSB) repair. Nevertheless, two additional explanations not mutually exclusive can be given, based on the scarce data available.

1. *Low level of adenosine triphosphate (ATP)*. Lowered level of ATP generation by mitochondria of oxidatively stressed cells may be the reason of lack of adequate energy supply necessary for the repair processes in the nucleus. Such adverse effect on DNA repair has recently been noted in cells treated with inhibitors acting at various sites of the mitochondrial electron transport (carbonyl cyanide 3-chlorophenylhydrazone, antimycin A, stigmatellin, rotenone and oligomycin) [13]. There is one more report supporting such explanation. Ogawa *et al.* [28] found an enhanced dysfunction of mitochondria estimated as ROS production in T lymphocytes pre-treated with hydrogen peroxide before X-irradiation as compared to that after X-irradiation alone. An additional factor was destabilization of lysosomal membranes and iron ion release, thus increasing ROS generation due to the Fenton reaction.
2. *Depletion of reduced glutathione (GSH)*. GSH depletion takes place *in vivo* [1] upon Ag NP treatment, but *in vitro* also a small increase has been noted at low NP concentrations [2, 15]. The effect of GSH depletion on SSB rejoining was a subject of studies 25 years ago ([27]; reviewed in [9, 31]). Rejoining of SSB was delayed and incomplete during a one hour's incubation period after oxic, but not after anoxic exposure of GSH-deficient cells, as reported by [31]. So, lowered GSH content may be responsible for the decrease in SSB repair rate.

Notwithstanding the molecular mechanism of the observed decrease in the rate of DNA break rejoining, this effect may have consequences for human health. It was shown recently in a technically advanced study [7] that the delay in DNA break rejoining increases the probability of mutagenic modifications. Hence, increased mutation frequency may take place, also in key regulatory genes, tumor supressors and protooncogenes that can potentially lead to cancer (review in [21]). In conclusion, in view of the increasing practical applications of nanomaterials, potential health hazard of Ag NP should be carefully evaluated, also with regard to carcinogenicity.

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