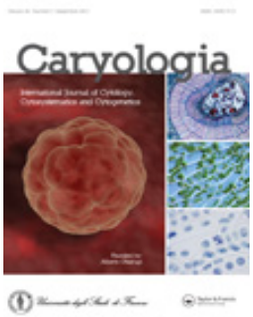

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Cytogenetic effects of chitosan-capped silver nanoparticles in the *Allium cepa* test

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Silver nanoparticles are one of the most commonly used nanomaterials. However, there remains insufficient information on their genotoxic effects. The goal of this study was to investigate cytotoxic and genotoxic effects of chitosan-coated AgNPs using the well-established *Allium* test. Root tip cells were treated with solutions at different concentrations (1, 2.5, 5 and 50 mg l^{-1}) of chitosan-coated AgNP (size: 10–30 nm; organic coat: 2–5 nm). On the same slide mitotic abnormalities, chromosome aberrations and micronuclei were detected. Also mitotic and phase indexes were analyzed. No cytotoxic or genotoxic effects were found at concentrations below 5 mg l^{-1}. The absence of induction of chromosomal and mitotic abnormalities by chitosan-capped AgNPs at low concentrations is possibly due to the capping, which may partly protect the cells from direct interaction with the AgNPs. Mitotic and chromosomal abnormalities and micronuclei were detected at a concentration of 50 mg l^{-1}. Significant increase in mitotic index was found at 5 and 50 mg l^{-1} concentrations of AgNP. The data demonstrated that chitosan-coated AgNP exhibit both clastogenic and aneugenic activity.

Keywords: *Allium* test; chromosome aberrations; genotoxicity; mitotic index; polyploidy; micronuclei; silver nanoparticles

Introduction

The nanotechnology industry is developing rapidly and many engineered nanomaterials have been used for their benefits without recognition of their harmful effects on human health and the environment (Singh et al. 2009).

Silver is one of the most frequently used nanomaterials due to its antibacterial properties. The antimicrobial activity of silver nanoparticles (AgNP) has many applications (Kim et al. 2007; Choi et al. 2008; Singh et al. 2009; Wijnhoven et al. 2009; Quadros and Marr 2010; Rai et al. 2012). In particular, the beneficial effect of AgNP on infection prophylaxis led to the development of medical devices (Chaloupka et al. 2010). However AgNP can be toxic not only for prokaryotic cells. Nanoparticles as well as other medicinal agents require toxicity and cytotoxicity testing to ensure their safety (Modallal et al. 2008; Aye et al. 2013; Hu et al. 2013). AgNP toxicity and genotoxicity has been found in different eukaryotic cellular models, such as human lung fibroblasts, human hepatoma cells (HepG2) (AshaRani et al. 2009; Kawata et al. 2009), rat alveolar macrophages (Carlson et al. 2008) or plant and algae cells (Navarro et al. 2008; Kumari et al. 2009; Ruffini Castiglione et al. 2009; Panda et al. 2011; Klancnik et al. 2011).

However, AgNP remains a controversial research area with regard to its genotoxicity and toxicity (Ghosh et al. 2012). Therefore extensive studies are required to understand the effect of AgNP on human health and the environment.

An important task of today's nanotechnology is searching for nanomaterials with safe biocompatible properties. It is known that the biological activity of nanoparticles depends on the size, shape and coat of nanoparticles, and if these properties change, the bio effects can differ dramatically. For example, coating (capping) has been shown to significantly reduce cytotoxicity (Derfus et al. 2004; Hoshino et al. 2004; Hardman 2006; Reijnders 2008).

Biological assessment with different markers is required in order to provide information that will allow the informed design of future nanomaterials, ensuring their biocompatibility and minimizing potential adverse health risks (Singh et al. 2009). Such knowledge is of great importance for nanotechnology to grow in a responsible and sustainable manner (Asare et al. 2012).

Plant bioassays are important tests in the detection of genotoxic and toxic agents and contamination in the environment (Adamakis et al. 2013; Dixit et al. 2013; Frescura et al. 2013). *Allium cepa* L. has been used to evaluate DNA damage, such as chromosome aberrations, micronuclei and disturbances in the mitotic cycle (Bakare et al. 2012; Frescura et al. 2012; Olorunfemi et al. 2012; Achary et al. 2013). Due to its sensitivity, the *A. cepa* test was the first of nine plants assay systems evaluated by the Gene-Tox Program of the US Environmental Protection Agency (Grant 1994). The *Allium* test is now frequently used for environmental monitoring (Fiskesjo 1993; Leme and Marin-Morels 2009), laboratory research (Pesnya and Romanovsky 2013) and has also bee pro-

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posed for assessing nanomaterials (Kumari et al. 2009; Klancnik et al. 2011).

Therefore, in the present study, genotoxic and cytotoxic effects of chitosan-coated AgNP on root meristematic cells of *Allium cepa* L. were investigated.

Materials and methods

Silver nanoparticles

The dispersion of chitosan-coated AgNP in deionized water, synthesized by an electrochemical method (Rodriguez-Sanchez et al. 2000) was a gift from SPA "Likom", Yaroslavl, Russia. According to the manufacturer, the concentration of AgNP in dispersion is 5 mg l^{-1}, density 270–300 nanoparticles/μl^3, size of nanoparticles 10–35 nm, and size of coat is 2–5 nm, spherical in shape, surface area 24.6 m^2 g^{-1}, purity: 99.5%.

Measurements of AgNP performed using scanning probe microscope FemtoScan (Moscow, Russia). The atomic force microscopy (AFM) method was used. Data obtained were processed by software Femtoscan Online (Advanced Technologies Center, Moscow, Russia; http://www.nanoscopy.net). The AFM images (Figure 1) obtained of AgNP revealed the particles to be spherical in shape, well distributed, with small aggregation, in the size range of 10–40 nm as specified by the manufacturer.

The stock dispersion of AgNP was serially diluted at different concentrations and vortexed (1, 2.5, 5 and 50 mg l^{-1}).

2.2. Allium *test and treatment*

As a method of analysis of genotoxic activity was used *Allium* test (Constantin and Owens 1982; Fiskesjo 1985).

Onion bulbs (*Allium cepa* L., $2n = 16$) of the Stuttgarten-Risen variety, average weight 25 g, were placed in small glass jars with their basal ends dipped in distilled water, and germinated at room temperature (24 ± 3°C). When the newly emerged roots were 0.50 cm in length, they were used in the test. Roots of *A. cepa* were treated with a series of concentrations of AgNP, i.e. 1, 2.5, 5 and 50 mg l^{-1} for 96 h in the absence of direct light. Control groups were treated with deionized distilled water. After treating root-tips were placed in a solution of ethanol (96%) and glacial acetic acid (3:1) for 48 h then washed with distilled water and dyed using aceto-orcein for 1 h. The squash technique was applied for the study of the mitotic index (MI) and phase indexes, mitotic and chromosomal aberrations and micronuclei. Five replicates (bulbs) were performed for each group and scoring was given from the three roots of each replicate (15 slides for each concentration) (Barbério et al. 2011). The MI was calculated for each treatment as a number of dividing cells per 700 cells and also scored the proportions of mitotic phases (Figure 2).

Chromosomal aberrations (chromatid (single) and chromosome (double) bridges and fragments) were scored in 100 ana-telophases per slide. Mitotic abnormalities (lagging chromosomes and polyploidy) were scored in 1000 mitotic cells per slide. Micronuclei frequency

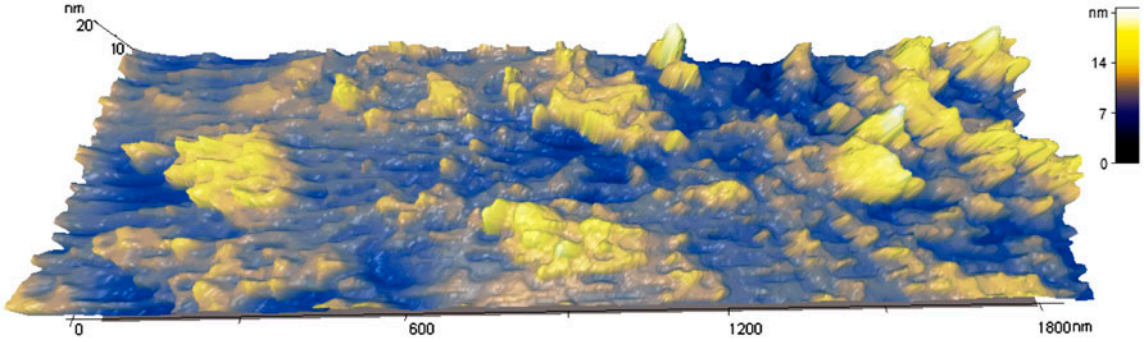

Figure 1. (Color online) Atomic force microscopy (AFM) image of AgNP.

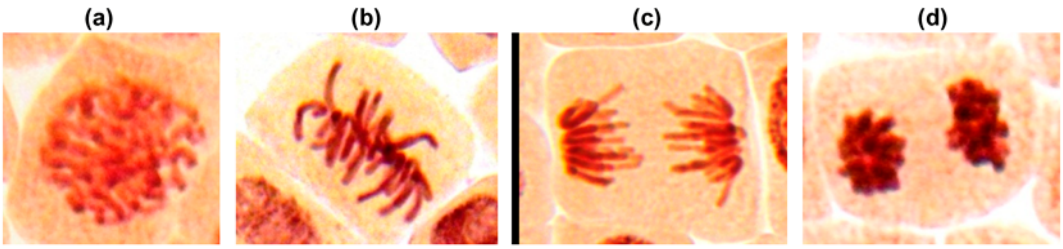

Figure 2. (Color online) Stages of mitosis in the meristematic cells of *A. cepa*: (a) prophase; (b) metaphase; (c) anaphase; (d) telophase.

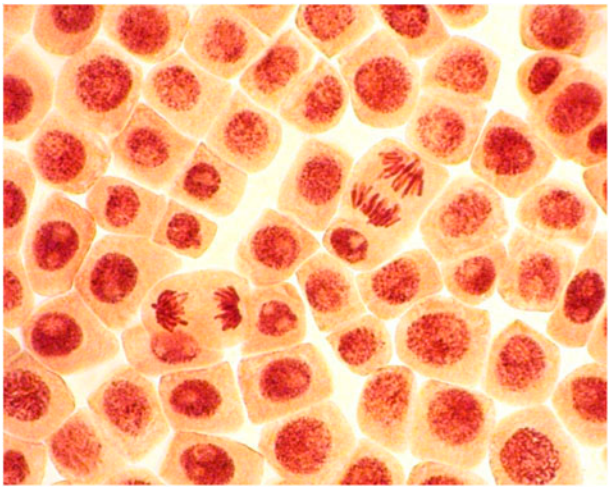

Figure 3. (Color online) Control meristematic cells of *A. cepa*.

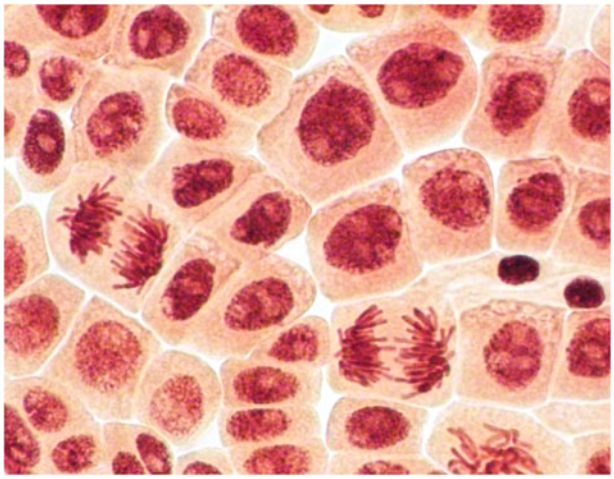

Figure 4. (Color online) Giant polyploid cells (anaphases and interphases) in root meristem of *A. cepa* after incubation in solution of 50 mg l^{-1} AgNP.

was expressed as the number of interphase cells with micronuclei per 3000 for every slide. All examinations were done under a light microscope at 400× magnification. The most frequent abnormalities (Figures 4, 5, 6) and normal cells (Figures 2, 3) are shown in photomicrographs. The results regarding MI and phase indexes and the frequency of mitotic and chromosomal abnormalities in root-tip cells of *A. cepa* are summarized in Tables 1–3.

2.3. *Statistical analysis*

Statistical calculations were done using Statistica 8.0. The differences in the mitotic index and phase indexes between treated and control groups were tested applying the non-parametric Mann–Whitney test. Frequencies of chromosome aberrations, micronuclei and

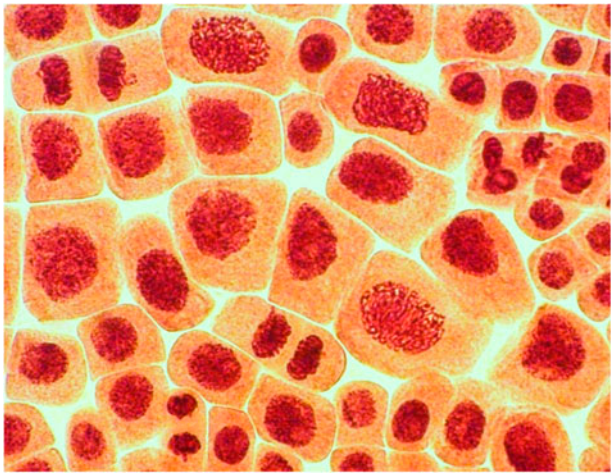

Figure 5. (Color online) Giant polyploid cells (prophases, telophases and interphases) in root meristem of *A. cepa* after incubation in solution of 50 mg l^{-1} AgNP.

mitotic abnormalities were statistically analyzed by Student's *t*-test and ANOVA. The level of significance was accepted at $p \leq 0.05$ (*).

3. Results and discussion

3.1. *Mitotic and phase indexes*

AgNP at concentrations 5 and 50 mg l^{-1} significantly increased the number of dividing cells in *A. cepa* root meristem. The most pronounced effect was registered at 50 mg l^{-1} concentration (Table 1). It should be noted that MI significantly higher than that in the control can be harmful to cells, leading to a disordered cell proliferation and even to the formation of tumor tissues (Leme and Marin-Morels 2009). Additionally, phase indexes are affected only at this concentration of AgNP. The prophase index and metaphase index are increased, while that of the anaphase index and telophase index are diminished (Table 1). According to Prokhorova et al. (2008), increasing frequency of prophases is associated with the violation of the chromosomal supramolecular structure. The situation when the frequency of metaphases increases, while that of anaphases and telophases decreases, can be associated with the action of AgNP on the achromatic spindle. In this case chromosomal segregation cannot occur, which may result in the appearance of genomic mutations (e.g. polyploidy and aneuploidy) (Prokhorova et al. 2008). Several mechanisms have been proposed for the occurrence of polyploidy (Zimmet and Ravid 2000; Gautam and Kumar 2013; Beyaz et al. 2013)

AgNP at 1 and 2.5 mg l^{-1} concentrations caused slight, but not statistically significant, increases in MI and prophase index (Table 1). Our results indicate that chitosan-capped AgNP can stimulate mitotic activity in plant tissue and disturb proportions of mitotic phases, which may result in mitotic abnormalities.

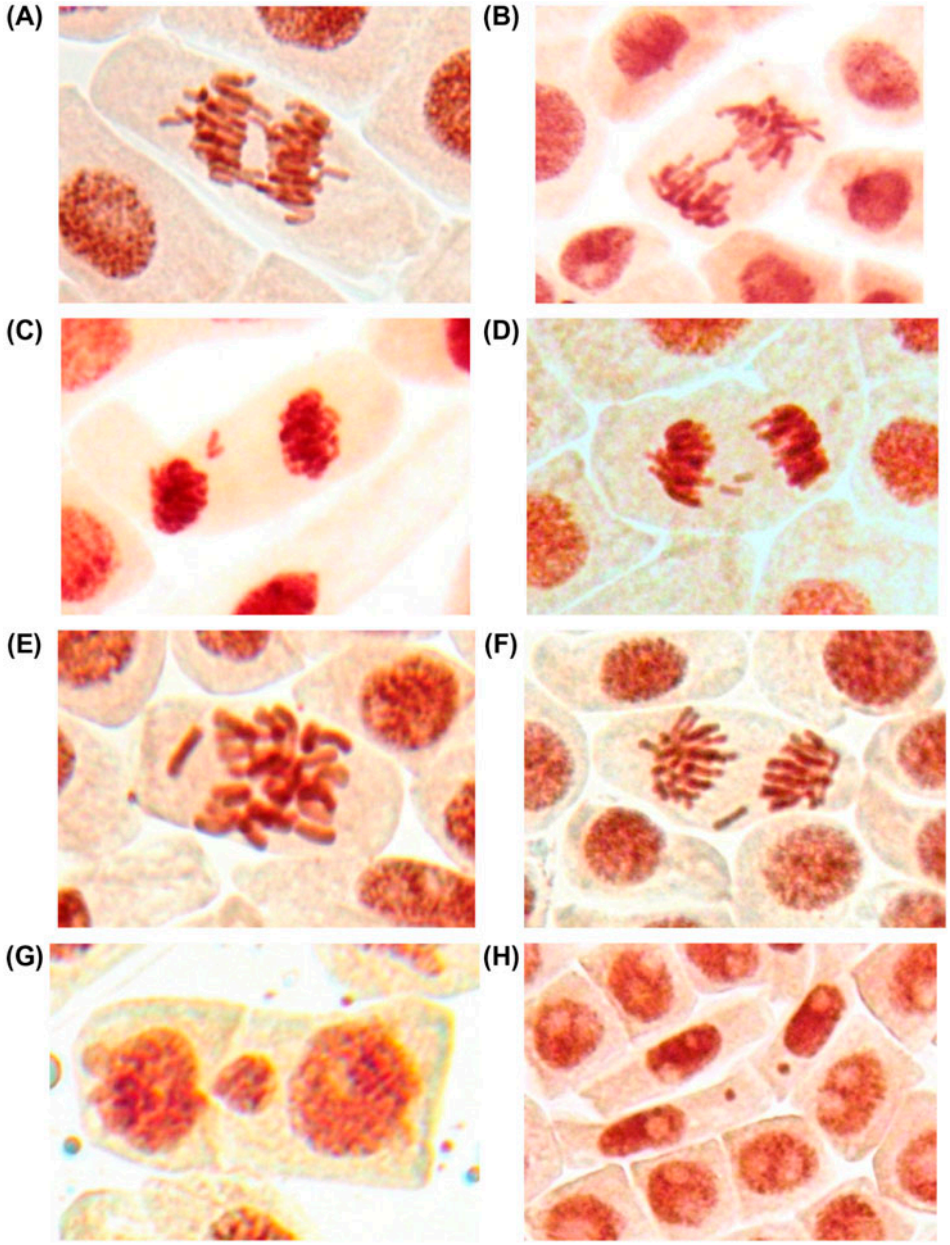

Figure 6. (Color online) Mitotic and chromosome abnormalities and micronuclei in root meristematic cells of *A. cepa*: bridges (A, B), acentric fragment (C), double fragments (D), lagging chromosomes (E, F) and micronuclei (G, H).

Table 1. Data on mitotic and phase indexes (mean ± SD) in the meristematic cells of *A. cepa* roots treated with solutions of silver nanoparticles.

AgNP concentration (mg l^{-1})	Mitotic index, %	Phase index, %			
		Prophase	Metaphase	Anaphase	Telophase
Control (0)	10.15 ± 0.38	53.39 ± 0.65	20.26 ± 0.72	13.06 ± 0.46	15.93 ± 1.26
1	10.55 ± 0.27	54.38 ± 1.25	19.14 ± 1.13	12.57 ± 0.87	13.91 ± 0.70
2.5	11.27 ± 0.40	53.69 ± 1.40	19.36 ± 1.24	12.91 ± 0.94	14.04 ± 0.79
5	12.18 ± 0.56*	55.28 ± 1.11	19.32 ± 0.55	11.74 ± 0.86	13.66 ± 1.05
50	17.20 ± 0.50*	62.55 ± 0.88*	25.45 ± 0.67*	5.06 ± 0.43*	6.95 ± 0.45*

*Statistically different when compared with the untreated control.

Table 2. Data on the number of mitotic disturbances (mean ± SD) in the meristematic cells of *A. cepa* roots treated by AgNP.

AgNP concentration (mg l^{-1})	Mitotic abnormalities, % per 1000 cells		
	Polyploidy	Lagging chromosomes	Total, %
Control (0)	0.00	0.05 ± 0.02	0.05 ± 0.02
1	0.00	0.02 ± 0.01	0.02 ± 0.01
2.5	0.00	0.03 ± 0.02	0.03 ± 0.02
5	0.03 ± 0.01*	0.05 ± 0.01	0.08 ± 0.02
50	5.45 ± 0.62*	0.27 ± 0.02*	5.72 ± 0.60*

*Statistically different when compared with the untreated control.

Mitotic abnormalities

In the control root tip cells were registered lagging chromosomes with frequency 0.05 ± 0.02% (spontaneous level). AgNP caused significant increasing in the frequency of lagging chromosomes at a concentration of 50 mg l^{-1} (Table 2, Figure 6E, F).

At concentrations of 5 and 50 mg l^{-1} polyploid cells were registered (Figures 4, 5). However, the frequency of polyploid cells was considerable only at 50 mg l^{-1}. Induction of polyploidy is considered to be the result of interference with components of the mitotic spindle during chromosome segregation or by blocking of cytokinesis (Honma et al. 2012). It is not clear whether chitosan coated AgNP produces polyploidy by a similar mechanism. It should be noted that observed polyploid cells did not look like classical colchicine induced polyploidy, according to Levan's (1938) micrographs. It is well known that colchicine has strong mito-depressive activity, while observed silver nanoparticles exhibit stimulating properties.

In addition, three or four nucleoli per nucleus were detected in the polyploid cells at interphase (Figure 4). Normally the diploid nucleus of *Allium cepa* contains one or two (Figure 3) nucleoli (sometime two nucleoli fuse into a single large nucleolus) (Wusheng et al. 1994; Panzera et al. 1996). It was established that the distribution of nucleoli number in an interphase nucleus can be used as an indirect practical method to distinguish diploid and polyploid cells (Dabrowska 1989). Thus, in the present study three or four nucleoli per nucleus may indicate polyploidy.

In the in the roots treated with 1 and 2.5 mg l^{-1} of silver nanoparticles polyploidy was not detected (Table 2).

Chromosomal abnormalities and micronuclei

Spontaneous frequencies of chromosomal aberrations (bridges and fragments) were 0.60 ± 0.16% and for micronuclei 0.011 ± 0.004 (Table 3).

Treatments with 1, 2.5 and 5 mg l^{-1} concentrations did not increase the frequencies of chromosomal abnormalities or micronuclei over the control values. The absence of induction of abnormalities by chitosan-capped AgNPs at low concentrations is possibly due to the capping, which may partly protect the cells from direct interaction with the AgNP (Nymark et al. 2012; de Lima et al. 2012; Ju et al. 2013). A significant increase in the frequency of chromosomal aberrations was observed only after exposure to 50 mg l^{-1} of AgNP. Bridges (Figure 6A, B) and fragments (Figure 6C, D) were registered in root meristems of *A. cepa* at this concentration. Additionally, significant increase in frequency of micronuclei (Figure 6G, H) was registered (Table 3). Micronuclei may arise mostly from acentric fragments or lagging chromosome (Fenech 2000). Fragments (Figure 6C, D) can be derived from chromosomal breakages caused by clastogenic effect or they may alternatively derive from chromosome aberrations, such as chromosomal bridges, which break up and originate acentric fragments (Fiskesjo 1993; Yi and Meng 2003; Leme and Marin-Morales 2008).These observations verify previous reports and show that AgNP can induce chromosomal aberrations and micronuclei in plant cells (Kumari et al. 2009; Panda et al. 2011). Additionally, new type of abnormalities (polyploidy) were registered (Table 2, Figures 4, 5). These findings indicate that organic-coated AgNP can affect the whole genome.

Thus, many engineered nanoparticles, including AgNP with different chemical properties, have been shown to be cytotoxic and genotoxic both to plant and mammalian cells (Kumari et al. 2009; Singh et al. 2009; Foldbjerg et al. 2011; Panda et al. 2011; Klančnik et al. 2011; Teodoro et al. 2011). AgNP are capable of entering the nucleus, and directly or indirectly interacting with nuclear material, leading to alterations in DNA

Table 3 Data on the number of chromosomal aberrations and micronuclei (mean ± SD) in the meristematic cells of *A. cepa* roots treated with colloidal solutions of silver nanoparticles.

AgNP Con. (mg l^{-1})	Chromosomal abnormalities, % per 100 ana-telophases			Micronuclei, % per 3000 cells
	Bridges	Fragments	Total, %	
Control (0)	0.40 ± 0.16	0.20 ± 0.11	0.60 ± 0.16	0.011 ± 0.004
1	0.27 ± 0.12	0.20 ± 0.14	0.47 ± 0.17	0.013 ± 0.004
2.5	0.40 ± 0.13	0.40 ± 0.24	0.80 ± 0.26	0.011 ± 0.004
5	0.60 ± 0.29	0.47 ± 0.23	1.07 ± 0.34	0.018 ± 0.006
50	2.47 ± 0.46*	2.13 ± 0.23*	4.60 ± 0.53*	0.118 ± 0.014*

*Statistically different when compared with the untreated control.

integrity (Kruszewski et al. 2011; Asare et al. 2012). Although the exact mechanism underlying genotoxicity of AgNP is yet to be elucidated, several studies have suggested that AgNP-induced DNA damage was apparently mediated through oxidative stress (Oberdorster 2004; Sayes et al. 2005; Reeves et al. 2008; Panda et al. 2011; Liu et al. 2012). Cellular interaction of AgNP which leads to the generation of reactive oxygen species has been shown to be related to the physicochemical characteristics of nanoparticles: size, coating, shape, surface charge (Carlson et al. 2008; Panda et al. 2011; Kim and Ryu 2013). Possible mechanisms for induction of oxidative stress by AgNP include direct generation of ROS from the surface of the particles, soluble compounds such as transition metals, and altered function of mitochondria or NADPH oxidase (Kim et al. 2011).

Conclusions

To summarize, the results of this study show that chitosan-coated AgNP did not exhibit cytotoxicity and genotoxicity at concentrations of 1 and 2.5 mg l^{-1}. Considerable genotoxic effects of chitosan-coated AgNP at concentration 5 and 50 mg l^{-1} were observed. The mitotic index was significantly increased at 5 and 50 mg l^{-1} concentrations of AgNP. However phase indexes were modified only at 50 mg l^{-1} concentration of AgNP. Polyploid cells were detected at 5 mg l^{-1} and 50 mg l^{-1} concentrations of AgNP. The total frequency of mitotic abnormalities (polyploidy, chromosome lagging), chromosome aberrations (fragments, bridges) and micronuclei were significantly increased at 50 mg l^{-1} concentrations of AgNP.

Thus chitosan-coated AgNPs exhibit both clastogenic and aneugenic activity in plant cells.

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Declaration of interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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