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The effects of silver ions and silver nanoparticles on cell division and expression of *cdc2* gene in *Allium cepa* root tips

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Abstract

The effects of silver nanoparticles (AgNPs), silver ions (Ag^+), and polyvinylpyrrolidone (PVP) on mitosis and expression of a gene encoding cyclin-dependent kinase 2 (*cdc2*) in onion roots were compared. Three concentrations (5, 10, and 15 mg dm^{-3}) were employed in combination with three incubation times (3, 6, and 9 h). PVP enhanced mitotic index and *cdc2* expression. Both silver forms decreased mitotic index and *cdc2* expression. Genotoxicity of both silver forms were indicated by three major distinguishable classes of chromosome aberrations: spindle disturbances, clastogenic aberrations, and chromosome stickiness. Concerning Ag^+ treatments, significant enhancements in occurrence of any chromosome aberration type was associated with significant decrease in mitotic index. On the other hand, disturbed spindle in AgNPs treatments was observed even in absence of significant reduction in mitotic index suggesting that AgNPs inhibit cellular events occurring during mitosis to proceed normally rather than starting of cell division.

Additional key words: chromosome aberrations, mitotic index, mitotic abnormalities, phase index, real-time PCR.

Introduction

The unique properties of nanoparticles, compared with their non-nanosized equivalents, attracted an increasing attention for their production and applications (Schmid 2010). Today, man-made nanoparticles, namely the engineered nanoparticles (ENPs), are used in different areas including electronics, medicine, pharmaceutics, industry, and agriculture (Bhushan 2010).

Silver nanoparticles (AgNPs) are one of the most widely used ENPs (Yu *et al*. 2013). The most common method used for AgNPs preparation involves reduction of a silver salt in the presence of polyvinylpyrrolidone (PVP) that serves as a stabilizer against agglomeration of the formed nanoparticles (Kittler *et al*. 2009). Compared with other silver forms, AgNPs possess distinguished antimicrobial properties (Shahrokh and Emtiazi 2009) that enhanced their regular use as antimicrobial agent in many industrial and medical products (Kim *et al*. 2007, Blaser *et al*. 2008, Klaine *et al*. 2008, Ma *et al*. 2010). Also, AgNPs are widely applicable in agriculture as pesticides (Park *et al*. 2006, Jo *et al*. 2009, Savithramma *et al*. 2012). All these applications of AgNPs make urgent necessity to study their impacts on biological systems.

Though there are several studies on the cytotoxicity and genotoxicity of AgNPs on mammalian and human cell lines, fewer studies were found to elucidate the effect of AgNPs on vascular plants. Depending on mitotic index, as an indicator of adequate cell proliferation, *Allium cepa* has been used by many researchers as a plant model system to evaluate the effects of many substances including AgNPs (Kumari *et al*. 2009, Pesnya 2013, Prokhorova *et al*. 2013). However, studies demonstrating the molecular bases underlying these effects are still lacking.

The mitotic cell cycle can be described simply as the duplication of chromosomes followed by their distribution between two daughter cells. Progress through the eukaryotic cell cycle is strictly controlled through several regulatory mechanisms such as reversible phosphorylation of regulatory proteins called cyclins (CYC). Phosphorylation is mediated through the activity of a family of cyclin-dependent protein kinases (CDKs) (John *et al*. 2001, Tank and Thaker 2011). Based on the putative cyclin-binding domains, CDKs are classified into eight classes: CDKA to CDKG and cyclin dependent kinases like (CKL). CDKA is the largest group among

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Abbreviations: Ag^+ - silver ion; AgNPs - silver nanoparticles; CDKs - cyclin-dependent kinases; CKL - cyclin dependent kinase like; CYC - cyclin; DW - deionized water; ENPs - engineered nanoparticles; LSD - least significant difference; PVP - polyvinylpyrrolidone; TEM - transmission electron microscopy.
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the plant CDKs, and they are encoded by a *cdc2* gene in onion (Tank and Thaker 2011). It is characterized with conserved PSTAIRE motif responsible for binding to CYC (Francis 2009). The *cdka* genes are expressed more or less constitutively both with respect to the cell cycle and different regions of the plant (Hirayama *et al.* 1991). The mitotic roles of CDKAs are indicated through their transit interaction with chromosomes during metaphase/ anaphase transition (Stals *et al.* 1997) and by localization closely to mitotic structures including preprophase band and spindle (Boruc *et al*. 2010). Hemerly *et al*. (1995) demonstrated the role of CDKAs in both G1/S and G2/M transitions.

In this investigation, we aimed to study the impact of Ag^+ and AgNPs in different concentrations and different incubation times on *cdc2* gene expression in relation to cell division in *Allium cepa* roots. Bearing in mind that AgNPs used in this study were PVP-coated particles, the impacts of PVP were also evaluated.

Materials and methods

Silver nanoparticles characterization and dispersion: AgNPs were commercially purchased from *Nanotech*, Cairo, Egypt, as PVP-coated particles suspended in deionized water (DW). Transmission electron microscopy (TEM) showed that particles were roughly spherical with an average size of 20 - 30 nm (Fig. 1 Suppl.). Experimental concentrations were prepared, just before use, by diluting of AgNPs stock solution (1 000 mg dm^{-3}) in DW and then sonicated at 100 W and 30 kHz for 30 min.

Plants and experimental conditions: Healthy bulbs of onion (*Allium cepa* L., 2n = 16) were kindly provided from the Field Crops Research Institute, Agricultural Research Centre, Giza, Egypt. Peeled bulbs were incubated in the dark at a temperature of 25 ± 1 °C in 50 cm^3 beakers for 48 h, and DW was renewed twice a day. Triplicates of onion bulbs having about 2.5 cm roots were selected for subsequent treatments, where DW was replaced by treatment solution.

A factorial experiment was conducted to investigate the effect of three concentrations (5, 10, and 15 mg dm^{-3}) of AgNPs in combination with three incubation times (3, 6, and 9 h). The effects of the same concentrations of both $AgNO_3$ and PVP were evaluated in two parallel experiments employing the same factorial design. The results were compared with a control treated with DW for the same time. Root tip samples were collected, washed thoroughly with DW, and blotted on filter paper.

Cytological analysis: Washed tips from each treatment were fixed in ethanol + glacial acetic acid mixture (3:1, v/v) for 48 h and finally stored in ethanol (70 %, v/v) at 4 °C. Just before examination, fixed root tips were hydrolyzed in 1 M HCl at 60 °C for 4 - 5 min, then placed in Feulgen stain for approximately 1 h. The intensely stained meristematic area was cut, squashed between slide and cover, and 2000 cells per treatment were examined by light microscope at 1 000 × magnification. To evaluate the effect of different treatments on cell division, mitotic and phase indices as well as percentages of different chromosome aberrations were calculated. Mitotic index was calculated as the percentage of dividing cells from total cells whereas phase indices and percentages of chromosome aberrations were calculated as percentages of dividing cells.

Real-time quantitative PCR: Total RNA was extracted from root tips using *Direct-zol™ RNA MiniPrep* (http:// www.zymoresearch.com). RNA samples were treated with DNase (*Fermentas*, Waltham, MA, USA) to get rid of residual genomic DNA. A purity and concentration of RNA was determined using a *Nanodrop* spectrophotometer (ND-2000c, *Thermo Fisher Scientific*, Wilmington, DE, USA). One µg of each RNA sample was utilized in cDNA synthesis with the aid of *SensiFAST™* cDNA synthesis kit (http://www.bioline.com). Quantitative amplifications of *cdc2* gene were performed using a *Mx3000P* (*Stratagene*, CA, USA) qPCR system with the specific primers: 5'-TTA TGGGTACCCCAAACGAA-3' and 5'-TTGTCCCAA GATCCCTGAAG-3'. Results were normalized to those of *actin* as control obtained upon utilization of the primers: 5'-GCTTCCCGATGG TCAAGTCA-3' and 5'-GGATTCCAGCTGCTTCCA TTC-3'. The amplification protocol was 95 °C for 10 min followed by 40 two-step cycles of amplification (95 °C for 15 s, and 60 °C for 60 s). Average expression recorded in roots treated with DW for 3 h was considered as a quantification unit.

Statistical analysis: Results of each treatment were expressed as mean of three replicates ± standard deviation (SD). For each investigated parameter, the least significant difference (LSD) at $P = 0.05$ was calculated using *SPSS v. 14* while regression analysis was performed using *Minitab v. 10.0* software.

Results

Mitotic index under control conditions was 10.75, 9.88, and 10.1 % for 3, 6, and 9 h treatments, respectively (Fig. 1). Compared with the corresponding controls, PVP had no significant effect on cell division except after

treatment lasted 9 h when a significant enhancement (16.5 - 20 % of controls) was recorded at all used concentrations. On the other hand, both silver forms reduced mitotic index in a dose- and time-dependent manner, the reduction was better pronounced using Ag^+. Except in the case of 5 mg dm^{-3} for 3 and 6 h treatments, significant reduction (19.7 - 70 % of controls) in mitotic index was observed upon the exposure to all Ag^+ treatments. Concerning AgNPs, the significant reduction (14.1 - 38.1 % of controls) was restricted to concentrations 10 and 15 mg dm^{-3} and treatments lasted for 6 and 9 h. The regression analysis showed a positive correlation between mitotic index and PVP concentration (Table 1 Suppl.). The cytotoxic effect of Ag^+ and AgNPs were confirmed by negative correlation between mitotic index and silver concentration or treatment duration (Table 1 Suppl.).

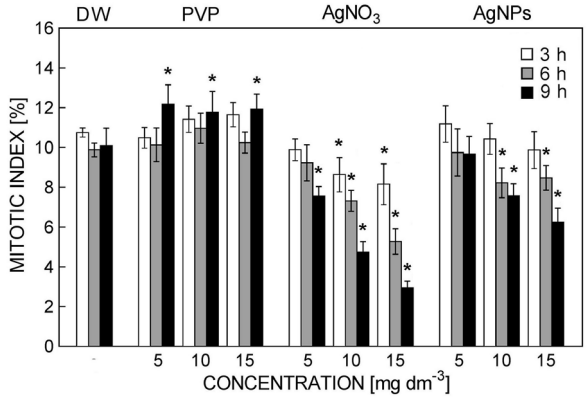

Fig. 1. Effects of treatments with polyvinylpyrrolidone (PVP), $AgNO_3$, and silver nanoparticles (AgNPs) at concentrations 5. 10, and 15 mg dm^{-3} lasted for 3, 6, and 9 h on mitotic index in *Allium cepa* root tips. Values are calculated as percentage of dividing cells. Means ± SDs, $n = 3$, * - significantly different compared with control (DW) at $P = 0.05$.

With regard to controls, the metaphase index significantly decreased upon exposure to 10 mg dm^{-3} PVP for 9 h while significant increase in the anaphase index was recorded upon exposure to 10 and 15 mg dm^{-3} PVP for the same treatment duration (Fig. 2 Suppl.). Otherwise, PVP had no significant effect on mitotic phase indices. Concerning both silver forms, regression analysis reflected general tendency to increase prophase and metaphase indices symbolized by positive correlations between each of these indices on one hand and silver concentration or treatment duration on the other hand (Table 1 Suppl.). Opposite trend was recorded for the remaining two phase indices.

Under control conditions, percentage of total chromosome aberrations was 5.72, 5.96, and 6.42 % of dividing cells for 3, 6 and 9 h treatments, respectively (Fig. 2). Compared with controls, PVP had no significant effect on occurrence of chromosome aberrations. On the other hand, genotoxic effect of silver appeared as significant increments in total chromosome aberration percentages that were more pronounced upon exposure to Ag^+ (44.5 - 194.3 % of controls) than to AgNPs (67 - 133.7 % of controls). For both silver forms, significant enhancements in percentage of total chromosome aberrations were always associated with significant decreases in mitotic index.

Three major classes of aberrations were distinguished (Fig. 3): *1*) spindle disturbances and its consequences (*e.g.*, C-metaphase, chromosome loss, multipolar anaphase, and star anaphase), *2*) clastogenic aberrations (breaks, bridges, and ring chromosomes), and *3*) chromosome stickiness. With respect to controls, PVP had no significant effect on occurrence of any of these classes while significant enhancements were recorded upon exposure to Ag^+ or AgNPs treatments (Fig. 2). Spindle disturbances were more common after AgNPs treatments while clastogenic aberrations and chromosome stickiness were better pronounced after Ag^+ treatments (Fig. 2). The genotoxic effects of both silver forms were confirmed by positive correlations between percentage of total chromosome aberrations and silver concentration or treatment duration. Similar correlations were recorded regarding different classes of chromosome aberrations (Table 1 Suppl.).

No significant changes were recorded in expression of the *cdc2* gene after treatment with different PVP concentrations for 3 h and 5 mg dm^{-3} for 6 h (Fig. 4). Otherwise, significant enhancements (25.5 - 50.5 % of controls) were recorded. By contrast, both silver forms reduced *cdc2* gene expression. Except in the cases of 5 mg dm^{-3} for 3 and 6 h, significant decreases (28 - 61.8 % of controls) in *cdc2* expressions were recorded at all Ag^+ treatments. Concerning AgNPs, significant decreases (34 - 52.5 % of controls) in *cdc2* expressions were restricted to 10 mg dm^{-3} for 9 h and 15 mg dm^{-3} for 6 and 9 h. Regression analysis revealed a positive correlation between expression of *cdc2* gene and PVP concentration and treatment duration. Negative correlations were recorded for Ag^+ and AgNPs treatments (Table 1 Suppl.).

Discussion

In the present study, PVP application for 9 h enhanced cell division as indicated by increased mitotic index. This can be attributed to the role of PVP in counteracting phenolics (Shimelis *et al*. 2015) normally produced by onion roots (Vu *et al*. 2012) and potentially able to reduce growth of plants (Rasouli *et al*. 2016). The accompanied increase in *cdc2* gene expression can be also attributed to the role of PVP against phenolics (Babaei *et al*. 2013).

Involvement of CDKAs, including CDC2 kinase, in G2/M transitions (Hemerly *et al.* 1995) gives further idea

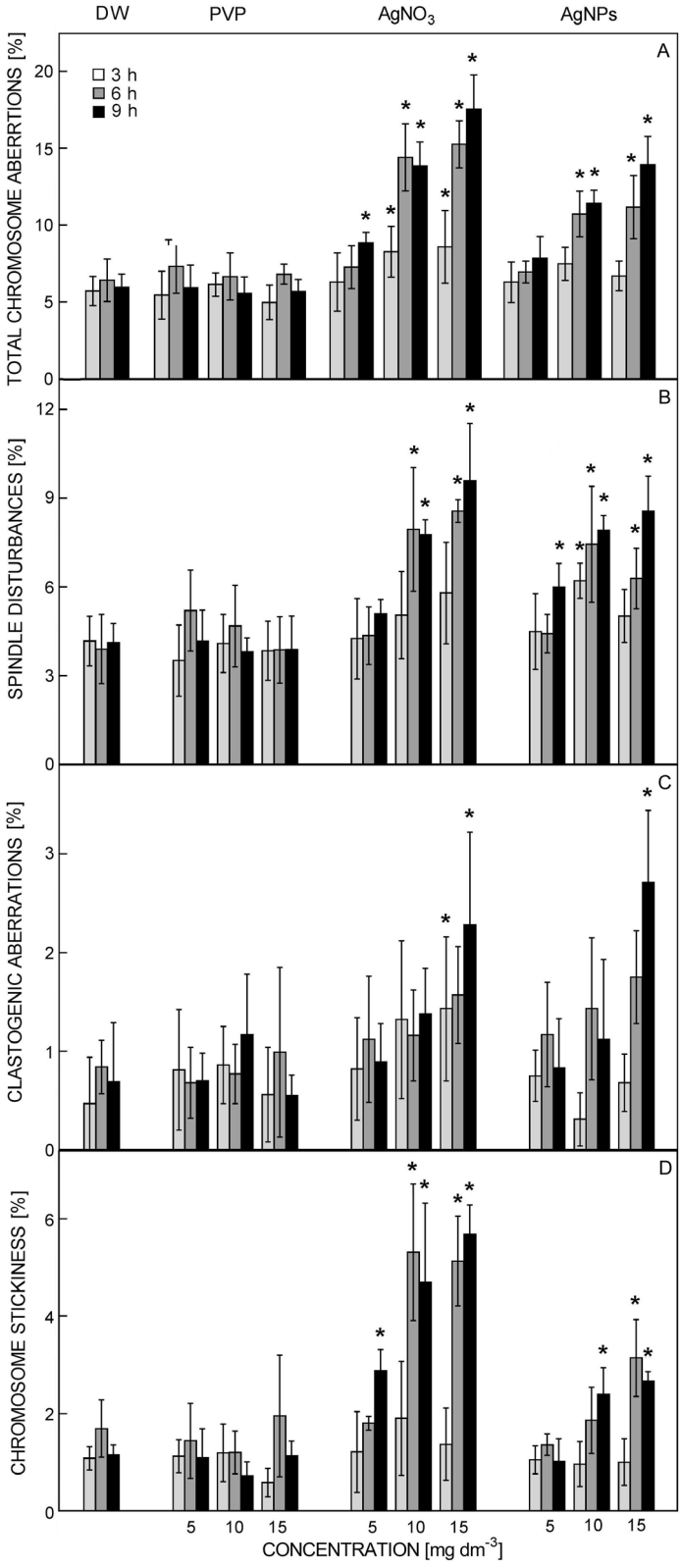

Fig. 2. Effects of treatments with polyvinylpyrrolidone (PVP), $AgNO_3$, and silver nanoparticles (AgNPs) at concentrations 5, 10, and 15 mg dm^{-3} lasted for 3, 6, and 9 h on chromosome aberrations in *Allium cepa* root tips: *A* - total abnormalities; *B* - spindle disturbances; *C* - clastogenic aberrations; *D* - chromosome stickiness. Values are calculated as percentage of dividing cells. Means ± SDs, $n = 3$, * - significantly different compared with control (DW) at $P = 0.05$.

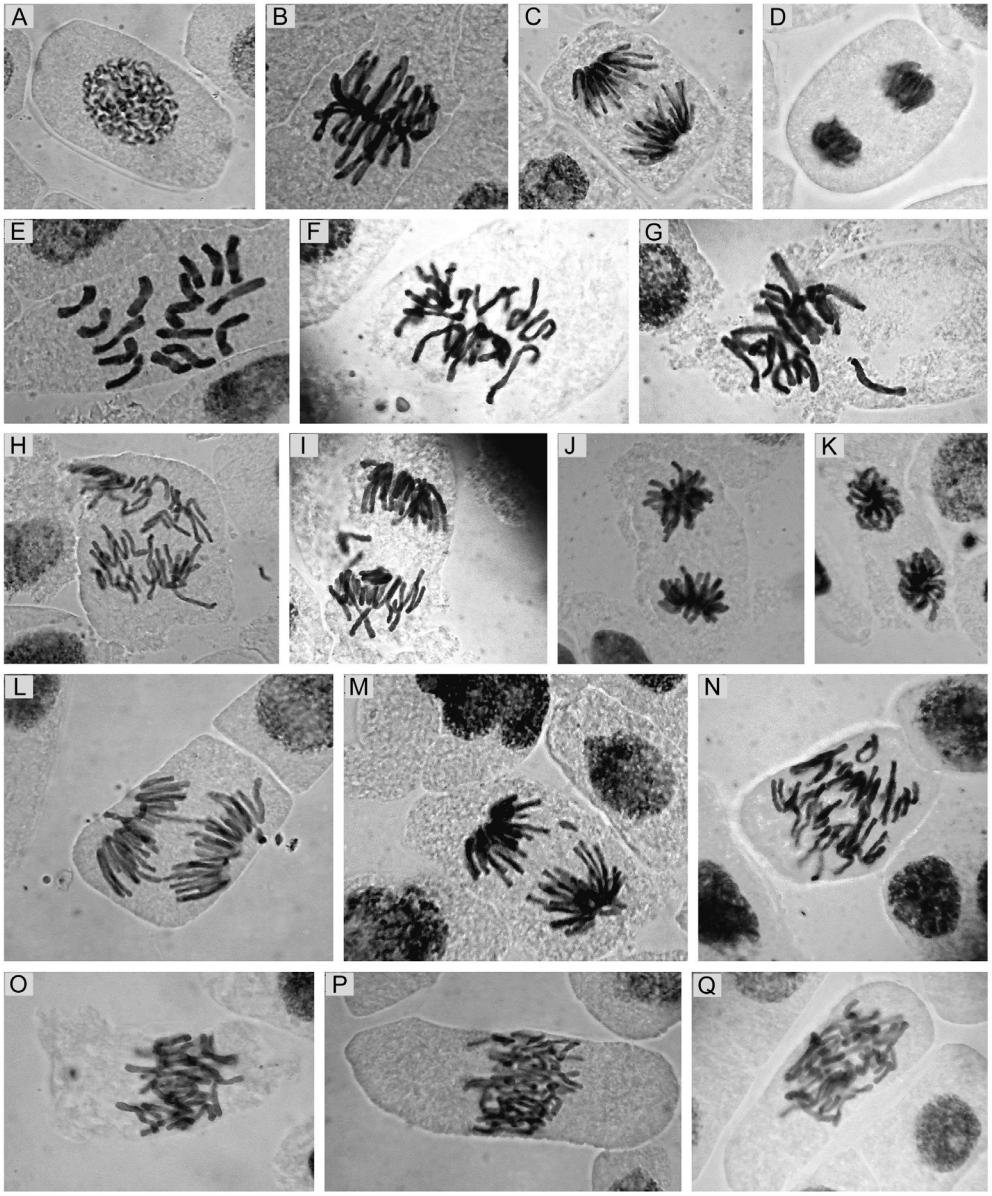

Fig. 3. Examples of normal and abnormal cell division in *Allium cepa* root tips following different treatments: *A*,*B*,*C*,D - normal prophase, metaphase, anaphase, and telophase, respectively, under control conditions (DW); *E* - c-metaphase after treatment with AgNPs (10 mg dm^{-3} for 6 h); *F*,*G* - metaphase with chromosome loss after treatment with AgNPs (15 mg dm^{-3} for 6 h); *H* - multipolar anaphase after treatment with AgNPs (15 mg dm^{-3} for 9 h); *I* - anaphase with chromosome loss after treatment with Ag^+ (15 mg dm^{-3} for 9 h); *J* - star anaphase after treatment with AgNPs (15 mg dm^{-3} for 9 h); *K* - star telophase after treatment with Ag^+ (15 mg dm^{-3} for 9 h); *L* - anaphase with bridge after treatment with Ag^+ (15 mg dm^{-3} for 9 h); *M* - chromosome break after treatment with Ag^+ (15 mg dm^{-3} for 9 h); *N* - ring chromosome after treatment with AgNPs (15 mg dm^{-3} for 9 h); *O*,*P* - chromosome stickiness after treatment with Ag^+ (15 mg dm^{-3} for 9 h); Q - chromosome stickiness after treatment with AgNPs (15 mg dm^{-3} for 9 h).

about the molecular mechanism underlying the observed increase in mitotic index. However, coupling of increase in *cdc2* expression and in mitotic index was missed in 6-h treatments. In this context, Hemerly *et al.* (1993) recorded that increase in *cdc2* expression always precedes cell division in *Arabidopsis* but not always coupled with it. The association between CDC2 kinase and spindle during mitosis (Boruc *et al*. 2010) may explain significant increments in anaphase index observed after 9-h treatment with PVP at concentrations 10 and 15 mg dm^{-3}.

Our experiments showed cytotoxic and genotoxic effects of both silver forms but with some differences between them. Both silver forms decresed mitotic index and increased occurrence of chromosome aberrations that were more obvious in the case of Ag^+. Similar results were recorded in *Lathyrus sativus* (Panda *et al*. 2016). The attenuated effect of AgNPs compared with Ag^+ may be explained by a protective role played by PVP coating

in reducing Ag^+ leaching from AgNPs (Nymark *et al*. 2013). Supporting such explanation, significant effects of AgNPs were absent in 3-h treatments that showed the necessity of some time for leaching enough Ag^+ from nanoparticles.

The negative effect of both silver forms on mitotic index can be explained in light of the associated decline in *cdc2* expression. Generally, the decline in expression of *cdk* genes is a common stress response (Kitsios and Doonan 2011) leading to cell cycle arrest, prolonged S-phase progression, or delayed entry into mitosis (De Veylder *et al*. 2007). Nevertheless, the association between decreased mitotic index and declined *cdc2* expression was not found in all AgNPs treatments that may suggest a unique molecular mechanism for each silver form. In this context, Kaveh *et al*. (2013) recorded the influence of silver form on gene expression in *Arabidopsis*. The authors observed similar response only in 34 % of genes differentially expressed in response to AgNPs and Ag^+. In contrast with a decrease in level of *cdc2* expression following some AgNPs treatments recorded in this study, Syu *et al*. (2014) recorded protein accumulations including cell-division-cycle kinase 2 in *Arabidopsis* upon treatment with AgNPs. This contradiction can be attributed to differences in characteristics of nanoparticles (Remédios *et al*. 2012).

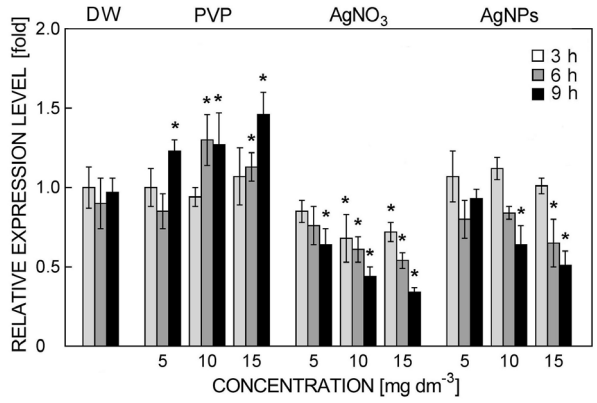

Fig. 4. Effects of treatments with polyvinylpyrrolidone (PVP), $AgNO_3$, and silver nanoparticles (AgNPs) at concentrations 5, 10, and 15 mg dm^{-3} lasted for 3, 6, and 9 h on *cdc2* relative expression in *Allium cepa* root tips. Values are relative to average expression recorded in roots treated with deionized water for 3 h. Means ± SDs, $n = 3$, * - significantly different compared with control (DW) at $P = 0.05$.

Modified cellular division phase indices and enhanced percentages of different types of chromosome aberrations were recorded after Ag^+ and AgNPs treatments. Increases in prophase index reflect a delay in chromosome condensation (Scolnick and Halazonetis 2000). Increase in metaphase index coupled with decrease in anaphase and telophase indices was a result of the accompanied spindle disturbance. AgNPs had a great impact on spindle and consequently metaphase/anaphase transition. Disturbance in spindle and its consequences upon applying AgNPs is a common observation (Kumari *et al*. 2009, Pulate *et al*. 2011, Pesnya, 2013, Panda *et al*. 2016). It is interesting to note that disturbed spindle after AgNPs treatments was observed even in absence of significant reduction in mitotic index. Concerning Ag^+ treatments, significant enhancements in occurrence of any chromosome aberration type was associated with a significant decrease in mitotic index.

Based on results of this investigation we conclude that the cytotoxicity, indicated with decrease in mitotic index, of both silver forms were differently related to *cdc2* expression. In addition, both silver forms have genotoxic impacts indicated by increase in occurrence of chromosome aberrations. However, cytotoxic and genotoxic impacts of Ag^+ were always coupled. Concerning AgNPs, genotoxicity appeared even in the absence of cytotoxic symptoms suggesting that AgNPs inhibit cellular events occurring during mitosis to proceed normally rather than starting cell division. In other words, AgNPs seems to be genotoxic rather than cytotoxic.

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