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A B S T R A C T

Nanotechnology a rapidly growing field during past decade, their wide spread use can cause serious health problems and several impact on environment. Among the various nanoparticles there is special concern regarding silver nanoparticles due to its wide use in various field, especially in agriculture sector. The present study aims to evaluate genotoxic and cytotoxic effect of synthetic silver nanoparticles on root tip cells of *Allium* species. Root tips of both species were treated with 10 nm synthetic silver nanoparticles of different concentration for three different time intervals. When the results were compared to control sample there was, decrease in mitotic index, increase in damaged cell and chromosomal aberration. Damaged cell includes concave plasmolyzed cell, cell wall deformities like blebs, breakage, elongated cell, whereas chromosomal aberration include ring, bridge, laggard, stickiness. It was found that 10 nm particle can enter through cell wall easily.

***Key words*:** Silver nanoparticles, *Allium cepa* L., *Allium fistulosum* L., Stathmokinesis, Concave plasmolysis

Nanoparticles are smarter particles whose size ranges in between 1 to 100 nm. Now a day's various engineered nanomaterials are being used in day-to-day activities from household to research. Among them nearly 25% are silver nanoparticles [40]. In agriculture sector AgNPs act as plant growth stimulator [35], for fruit ripening [34], [42] and as a fungicide [1]. They also show antimicrobial properties so being exploited in various consumer products like face cream, deodrants, in the sport socks, packaging of food material and also in cleaning solution [4]. Increasing incorporation of silver nanoparticles in various products also increases the chance of exposure to human as well as environment and consequently there would be bioaccumulation and biomagnification. Their presences in water were observed through the analysis of waste water from sewage treatment [15]. The porous network of cell wall of root cell act as natural sieve which allow the passage of small size AgNPs whereas large size nanoparticles are sieved out [38]. As, the small size silver nanoparticles enter it has also capability to increase the size of pore as a result large size nanoparticles can also enter through the cell wall [9]. Silver nanoparticles can cross the plasmodesmata pores of 50-60 nm in diameter [29], [14], [26]. Geisler et al. showed that AgNPs can aggregate in plasmodesmata and cell wall of *Arabidopsis* [13]. In addition to root and plasmodesmatal uptake, stomatal uptake of AgNPs were observed in *Arabidopsis* [12]. Li *et al*. showed that silver nanoparticles accumulation is 17-200 time more in foliar than root [24]. Through long distance transport AgNPs can be transported to leaves and other organs after entering into vascular tissue in crops [6], [29], [12]. So, it is possible that edible part of plant such as seed, fruit can get contaminated by AgNPs through translocation. Remarkable changes in the morphology of plants were observed after the exposure of AgNPs. Various parameters in plants were considered for assessing the phytotoxicity of AgNPs like growth potential, seed germination, biomass and leaf surface area in *Arabidopsis*. Toxicity on seed germination, biomass accumulation, root and shoot growth by AgNPs were reported in various plant species like *Arabidopsis* [33], *Phaseolous radiates* and *Sorghum bicolor* [23], rice [8], wheat [43] etc. Studies showed that AgNPs can cause cell aberration with alteration of cell structure and cell division [44]. That is why different types of aberration were observed in *Allium cepa* like abnormal metaphase, stickiness, chromatin bridge and cell disintegration and significantly decrease the mitotic index and impaired cell division [21]. In the root tips of *Vicia faba* L. AgNPs treatment decreased the mitotic index and increases the moicronuclei formation, chromosomal aberrations [32]. To observe the toxicity caused by mutagen, cytotoxicity and genotoxicity test can be done on plant cells. The *Allium* root tip cells chromosomal aberration assay is an established bioassay. It is validated by the International Programme on Chemical Safety (IPCS, WHO) and United Nations Environment Programme (UNEP), in methodical calibration for chemical screening and laboratory monitoring of genotoxicity caused by environmental agent [16]. Among all the material *A. cepa*, *A.*

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proliferum, A. fistulosum, Vicia faba have proved to be favoured material [17].

MATERIALS AND METHODS

Nanoparticles

Silver nanoparticle suspension was procured from Sigma Aldrich, the manufactured characteristic of particles is, size 10 nm, purity 99.7%, concentration 0.02µg/ml. For experiment three different concentration i.e., 20 ppm, 10 ppm, 5ppm were prepared.

Test system and treatment

The silver nanoparticles of different concentration 20ppm, 10ppm, and 5 ppm were prepared from the stock solution. For experiment healthy seeds of *Allium cepa* and *Allium fistulosum* were collected from ICAR Plandu, Ranchi and IARI, New Delhi respectively. Seeds were soaked in double distilled water for 9 to 10 hours. Soaked seeds of both species were kept on moist filter paper for germination in petri dishes separately. Immediately after germination transferred to the different concentration of silver nanoparticles for different time interval as mentioned in tables.

Microscopic examination

Root tips of proper size around 1-2 cm were cut with sterilized blade and fixed in Carnoy's fixative (i.e., 1:3 acetoalcohol) for 24 hours then transferred in 70% alcohol for preservation. For cytological study slides were prepared by Squash technique, using 1.5% acetocarmine as a stain. Slides were observed under Magnus s/n: C197050239 microscope and photographs were taken in 40X and 100X.

RESULTS AND DISCUSSION

The duration dependent effect of different concentration of 10 nm silver nanoparticles on cell division and chromosome behavior of *A. cepa* L. and *A. fistulosum* L. mentioned in (Table 1-2). The whole experiment was carried out in five replicates. The mitotic index (MI) and total abnormality percentage (TAB) were calculated using following formula's:

$$\text{Mitotic index (MI)} = \frac{\text{Total number of dividing cell}}{\text{Total cell count}}$$

$$\text{Total abnormality (\%)} = \frac{\text{Total number of abnormal cell}}{\text{Total cell count}}$$

Table 1 Effect of different concentration of 10 nm AgNPs on mitotic abnormalities in root tip of cells of *Allium cepa* L.

AgNPs		TCC	NCD	M.I%±SD	TAC	BB	BD	CP	LG	MN	NPB	PC	SK	SM	Abn%±SD
	Control	3000	521	17.36±1.23	-	-	-	-	-	-	-	-	-	-	-
20ppm	1hr	3000	361	12.03±1.46	53	6	10	2	12	3	1	2	3	15	1.75±0.57
	3hr	3000	348	11.60±0.83	58	4	12	3	8	5	2	1	5	18	1.92±0.52
	5hr	3000	321	10.70±2.57	67	7	16	2	11	9	1	5	2	14	2.23±0.45
10ppm	1hr	3000	447	14.90±1.11	37	3	9	1	7	2	2	1	2	10	1.26±0.42
	3hr	3000	417	13.90±1.87	40	2	5	2	8	6	3	3	4	7	1.35±0.44
	5hr	3000	406	13.53±2.54	42	2	8	2	10	4	1	1	3	11	1.42±0.45
5ppm	1hr	3000	461	15.36±1.77	20	2	3	1	4	3	0	1	1	5	0.66±0.32
	3hr	3000	461	15.36±1.25	24	1	3	1	7	1	1	1	2	7	0.75±0.43
	5hr	3000	457	15.23±1.18	26	1	5	1	3	2	2	2	4	6	0.81±0.98

Table 2 Effect of different concentration of 10 nm AgNPs on mitotic abnormalities in root tip of cells of *Allium fistulosum* L.

AgNPs		TCC	NCD	M.I%±SD	TAC	BB	BD	CP	LG	MN	NPB	PC	SK	SM	Abn%±SD
	Control	3000	561	18.7±0.57	0										
20ppm	1hr	3000	402	13.4±2.03	63	3	13	4	12	2	2	3	6	18	2.07±0.61
	3hr	3000	381	12.7±1.48	70	5	11	5	19	4	1	6	4	15	2.24±0.59
	5hr	3000	351	11.7±1.43	72	8	8	6	18	2	1	1	9	19	2.38±0.53
10ppm	1hr	3000	460	15.3±1.73	44	4	8	1	11	5	2	1	3	9	1.62±0.49
	3hr	3000	442	14.7±1.36	56	6	13	7	10	3	1	3	2	11	1.85±0.51
	5hr	3000	423	14.1±1.27	61	1	19	9	16	1	2	1	6	16	2.01±0.42
5ppm	1hr	3000	511	17.03±1.76		2	3	1	5	1	0	0	1	7	0.67±0.33
	3hr	3000	494	16.46±1.82		4	6	4	5	2	0	1	3	3	0.92±0.96
	5hr	3000	487	16.23±1.79		3	8	3	7	0	2	1	1	6	1.03±0.98

TCC- Total cell count, NCD- Number of cell division, TAC- Total abnormal cell,BB- Bleb, BD- Bridge, CP- concave plasmolysis, LG- Laggard, MN- Micronuclei, NPB- Nucleoplasmic bridge, PC- pulverized cell, SK- Stathmokinesis, SM- Sticky metaphase, Abn- Abnormality, ± - Standard Deviation

The result of experiment showed that silver nanoparticles can cause clastogenic, aneugenic and non clastogenic (physiological) aberration while no aberration was observed in control. The dose/duration is inversely proportional to M.I i.e., with increase in dose/duration M.I decreases while there is linear relationship with chromosomal aberrations i.e., with increase in dose/duration chromosomal aberration increase. The various abnormalities showed that AgNPs can act as clastogen that inducing disruption and breakage of chromosomes that can cause structural changes as well as aneugenic i.e., numerical changes whereas non-clastogen that causes physiological change. The various chromosomal abnormalities were observed in cells as the cells progressed in mitosis, cells at interphase or very early prophase showed hypo and hyperchromatization, karyopycnosis followed by karyorrehxis the initial stages of apoptosis. Plasmolysis and bleb were observed in higher concentration.

Metaphase studies

At metaphase the sticky chromosome at equatorial plate were found with excessive chromosome clumping (Fig 5). Metaphasic chromosome fragmentation, and ring formation were also observed. Metaphase with a group of unaligned chromosomes (Fig 6) was observed.

Anaphase studies

The most common anomalies observed at this phase were occurrence of chromatid bridge and laggard (Fig 9). Numerous different types of anaphase bridge were encountered i.e., single bridge, multiple bridge (Fig 8), ring (Fig 10). Occurrence of different types of bridge is the characteristic feature of this experiment. Precocious movement of chromosome, fragmented anaphase chromosome (Fig 11) also induced by the 10 nm synthetic silver nanoparticles of 20 ppm in 3 and 5 hours.

Telophase studies

Multiple laggards, bridge (Fig 12) and precocious movement of chromosome is the most common anomalies at telophase. Disorientation of chromosome at telophase were observed in all concentrations.

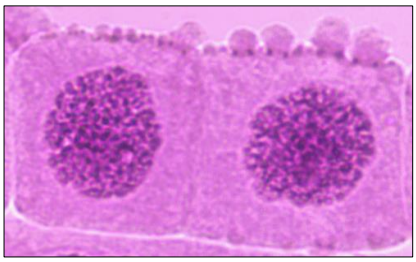

Fig 1 Blebs

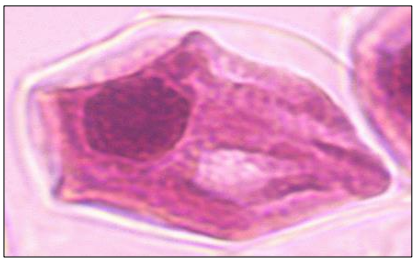

Fig 2 Concave plasmolysis

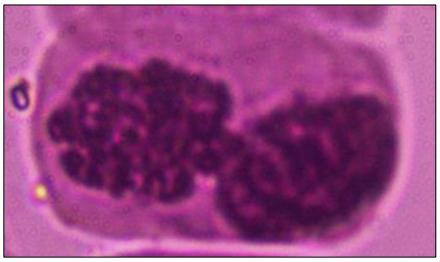

Fig 3 Nucleoplasmic bridge

Fig 4 Stathmokinesis

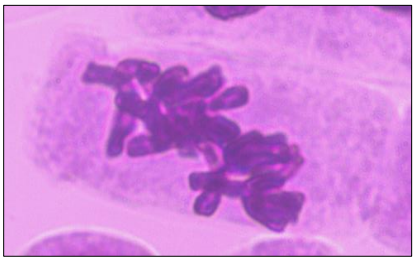

Fig 5 Sticky metaphase

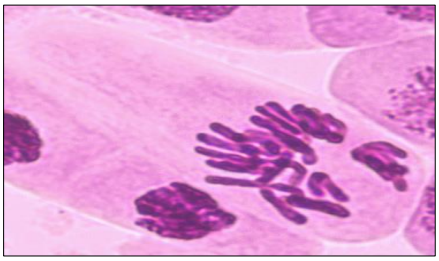

Fig 6 Abnormal metaphase with a group of unaligned chromosomes

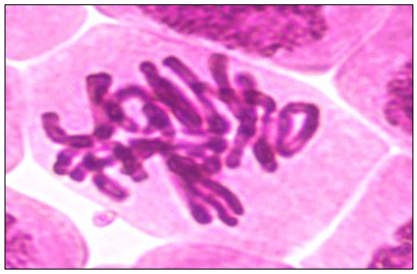

Fig 7 Ring at metaphase

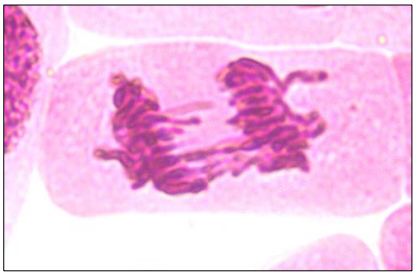

Fig 8 Thick anaphase bridge

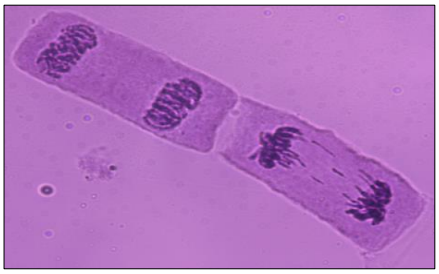

Fig 9 Anaphase with multiple laggard

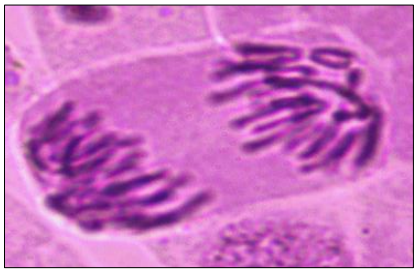

Fig 10 Ring at anaphase

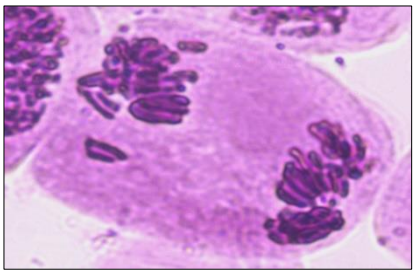

Fig 11 Abnormal anaphase with broken chromosome

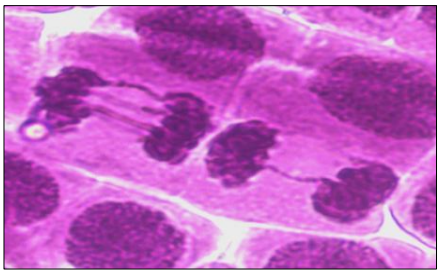

Fig 12 Single and multiple bridge at telophase

The chromosome clumping leading to sticky metaphase and anaphase bridge and stathmokinesis (Fig 4) are possibly due to the effect of the AgNPs in breaking the protein moiety of the nucleoprotein. In 1959 Venema [41] showed that these abnormalities are due to the disturbed RNA synthesis that causes interruption of protein metabolism. Pulverized chromosomes were observed in both 10ppm and 20ppm concentration of AgNPs which are the most common features in the present study. Bleb (Fig 1) is bulge of plasma membrane of cell formed due to cytoskeleton damage caused by intracellular pressure generated in the cytoplasm. Chromosome stickiness mostly observed at metapahse (Fig 5) and few at anaphase, may arises due to effect of AgNPs on nucleic acid causing intense polymerization of nucleic acid [18]. Anaphase bridges (Fig 8) are DNA thread between two DNA clump during anaphase segregation. It arises due to non-protein source of cohesion between sister chromatids that forms sister chromatid intertwines (SCIs) during replication process. SCIs is resolved in S phase but persistent unresolved SCIs can form anaphase bridge in mitosis [3]. The number of anaphase bridges increases in the presence of stress and that may be the reason of formation of characteristic anaphase bridges in my experiment because as concentration of AgNPs increase the stress of cell's internal environment increases. The BFB (Breaking-Fusion-Bridge) model of anaphase bridge formation is different from UFBs (Ultra- fine bridges) formation in which cleavage occurs

shortly after anaphase onset and is independent of cytokinesis [3], [7]. Presence of laggards at metaphase, anaphase and telophase is due to the delayed terminalization of spindle fibre. The fragment which appeared in between bridges is result of pulling the chromosomes towards the poles by spindle fibres, leading to chromosomal breakage and deletion [19]. Ring formation at metaphase (Fig 7], anaphase (Fig 10) was observed which might be due to improper attachment of kinetochore with spindle leading to joining of ends [20] or it may result from two terminal breaks in both chromosomal arms followed by fusion of proximal broken ends or by fusion of dysfunctional telomere of the same chromosomes because shortening of telomeric DNA repeats leads to the detachment of protective proteins from the chromosomal ends. In most of the case other than bridge and laggard, stathmokinesis was the most common feature, observed in many cells in which chromosomes are scattered throughout that may be due to delayed or disruption in nucleation of spindle fibres. Less plasmolyzed cells were frequently observed in all three concentrations, but in 20 ppm characteristic concave plasmolysis (Fig 2) was observed in which plasma membrane separates from the cell wall by the formation of concave pockets that may be due to change in the viscosity of cytoplasm. Heavy metal and other stress factors also affect the plasmolysis process, so plasmolysis test can also be used to test the cellular viability [11], [27], [36]. According to Fernandes et al. aneugenic agents promote complete inactivation of mitotic cycle which in turn may generate alteration such as polyploidy, multinucleated and micronuclei cells [10]. In 1974 kuriyama and sakai showed that the mitotic cycle is impaired by the interaction between AgNPs and tubulin-SH group [22]. Chromosome breakage (Fig 11) indicates clastogenic potential of AgNPs [9], which may cause the loss of genetic material [5]. Treatment of plant cell with AgNPs can produce excess ROS which can increase the oxidative stress causing lipid peroxidation and damages cell membrane permeability and cell structure, directly damaging protein, DNA and consequently result in cell death [2], [45]. Study reveals that nucleoplasmic bridge (Fig 3) increased significantly in dose related manner after exposure to ROS generated by activated neutrophils, superoxide and hydrogen peroxide [39]. As AgNPs increases the ROS, therefore occurrence of NPB may be the toxicity of AgNPs. Chromosomes of *Allium cepa* and *Allium fistulosum* showed almost similar effects in all different dose and duration. Among various of clastogenic and non clastogenic aberrations caused by AgNPs, nucleoplasmic bridge, formation of bleb, concave plasmolysis, ring at metaphase, anaphase and thick anaphase bridge were observed first time as far my knowledge obtained through reviewing papers.

CONCLUSION

Silver nanoparticles of 10 nm size impede the different stages of M-phase. In comparison to the control, variation in M.I and chromosomal aberrations were observed. Both these effects are duration and concentration dependent. In our studies silver nanoparticles of 10 nm size showed potential clastogenic and aneugenic effects in all concentration (5ppm, 10ppm, 20ppm), but maximum in 20 ppm. The M.I decreased from the control with increase in the concentration from 5ppm to 20ppm. The significant chromosomal aberrations were observed in anaphase like different types of bridges and metaphase as well as anaphase ring. The frequency of NPB gradually increases in root tips of *Allium* species from lower to higher concentrations of AgNPs. My observation and data help to access the potential toxicity of synthetic silver nanoparticles. My finding suggests that application of AgNPs in agriculture practices is of great concern, because the potential risk of mutagenesis is inescapable.

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