

## Evaluation of Genotoxicity by Micronucleus Assay *in vitro* and by *Allium cepa* Test *in vivo*

Christina N. Banti\* and Sotiris K. Hadjikakou\*

Section of Inorganic and Analytical Chemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece

\*For correspondence: [cbanti@uoi.gr](mailto:cbanti@uoi.gr); [shadjika@uoi.gr](mailto:shadjika@uoi.gr)

**[Abstract]** The *in vitro* and *in vivo* genotoxicity of new metallodrugs either as Small Bioactive Molecules (SBAMs) or Conjugates of Metals with Drugs (CoMeDs) is evaluated by the micronucleus test and the *Allium cepa* assay, respectively. Fetal lung fibroblast cells (MRC-5), normal human corneal epithelial cells (HCEC) and immortalized human keratinocytes cells (HaCaT) were incubated with solutions of SBAMs or CoMeDs at their IC<sub>50</sub> values for 48 h (the concentration of a compound which is required to inhibit the cells growth by 50% in relation to the non-treated cells). The micronucleus abundance percentage towards the corresponding one, of the non-treated cells indicates the *in vitro* genotoxicity of the formulations. The *in vivo Allium cepa* test comprises the exposing of the plant *Allium cepa* roots to an SBAMs or a CoMeDs solution for 48 h. The percentages of the mitotic index, the chromosome aberrations, the nuclear abnormalities and the presence of the micronucleus are calculated indicating the *in vivo* genotoxicity of the agent.

**Keywords:** Biological inorganic chemistry, Genotoxicity, Micronucleus, *Allium cepa*, Mitotic index, Metallodrug, Compound

**[Background]** Inorganic biochemistry, biological inorganic chemistry or bioinorganic chemistry is a multidiscipline scientific field which combines biological inorganic chemistry, biochemistry, biology, medicinal chemistry, microbiology and other fields (Latsis *et al.*, 2018). Nowadays, the research on the design and development of new metallodrugs [Small Bioactive Molecules (SBAMs) or Conjugates of Metals with Drugs (CoMeDs)] against numerous cancer types, includes their biological activity towards cancerous and non cancerous models (Banti *et al.*, 2016; Sainis *et al.*, 2016; Banti *et al.*, 2018; Chrysouli *et al.*, 2018a and 2018b; Latsis *et al.*, 2018; Milonis *et al.*, 2018; Stathopoulou *et al.*, 2018; Banti *et al.*, 2019; Polychronis *et al.*, 2019). Especially, the development of new SBAMs or CoMeDs imposes the need to assess their potential *in vitro* and *in vivo* toxicity against experimental models in order to check their potential risks (Banti *et al.*, 2016; Sainis *et al.*, 2016; Banti *et al.*, 2018; Chrysouli *et al.*, 2018a and 2018b; Latsis *et al.*, 2018; Milonis *et al.*, 2018; Stathopoulou *et al.*, 2018; Banti *et al.*, 2019; Polychronis *et al.*, 2019). Therefore, the micronucleus assay has been developed in monitoring genetic damage in normal human cells as it is a sensitive tool for toxicity screening and it is capable in reducing the use of animals in toxicological testing (Sahu *et al.*, 2014). The presence of micronucleus (MN) is a biomarker of mutagenic, genotoxic, or teratogenic agent influence (Torres-Bugarin *et al.*, 2014). The *in vitro* genotoxicity is calculated by checking the micronucleus frequency, upon treatment of normal cells by an agent at the concentration of its IC<sub>50</sub> value (Chrysouli *et al.*, 2018a and 2018b;

Latsis *et al.*, 2018; Milionis *et al.*, 2018; Sainis *et al.*, 2016; Stathopoulou *et al.*, 2018). The *Allium cepa* test, on the other hand, is an *in vivo* experimental model which is employed to evaluate the potential genotoxicity *in vivo*. *Allium cepa* displays high correlation with mammal test systems (Sainis *et al.*, 2016; Chrysouli *et al.*, 2018a and 2018b; Milionis *et al.*, 2018; Stathopoulou *et al.*, 2018). The assay is useful to identify the damaging effect or to evaluate the influence of a metallodrug at different concentrations (Sainis *et al.*, 2016; Chrysouli *et al.*, 2018a and 2018b; Milionis *et al.*, 2018; Stathopoulou *et al.*, 2018). The mitotic index (%) and DNA damages such as chromosomal aberrations (%), nuclear abnormalities (%) and micronucleus (%) are calculated when roots of *Allium cepa* are treated with different concentrations of a metallodrug for 48 h.

## **Materials and Reagents**

### **A. Micronucleus assay**

1. Latex gloves (KCWW, Kimberly-Clark, catalog number: 57330)
2. Pipette tips (Sterile)
3. 100 mm Petri dishes (SARSTEDT, catalog number: 83.3902), mammalian cell culture containers for adherent cells  
*Note: Any other Petri dishes or flask suitable for your cell line may work.*
4. Microscope slides (UltiDent Scientific, catalog number: 170-7107A)  
*Note: Any microscope slide that fits on your microscope can be used.*
5. Cover glass (UltiDent Scientific, catalog number: 170-C1818)  
*Note: Any cover glass can be used. The best cover glasses are the #1.5; however #1.0 or #0 may be used if high quality images are not required.*
6. 6-well tissue culture treated plate (Corning, catalog number: 3506)
7. 24-well tissue culture plate (Corning, catalog number: 3524)
8. Mammalian non cancerous adherent cells, for example, MRC-5, HCEC, HaCaT cells, *etc.*
9. Required complete culture media
  - a. DMEM (WISSENT, catalog number: 319-005-EL)  
*Note: The growth medium is adapted for each cell line. Verify with the cell line suppliers to determine the optimal growth medium for the cells and the necessary supplements.*
  - b. Fetal bovine serum (FBS) (WISSENT, catalog number: 095150)  
*Note: Usually, a 10% v/v solution is used, but some cell lines will require different concentrations. See with your cell line supplier.*
  - c. Penicillin/streptomycin (WISSENT, catalog number: 450-201-EL)  
*Note: It is usually supplied as a 100x stock and therefore must be diluted 1:100 in DMEM.*
  - d. L-glutamine (Sigma-Aldrich, catalog number: G3126)
10. Trypsin (0.05% or 0.25% in PBS) (Thermo Fisher Scientific, Gibco™, catalog number: 25200072)
11. Phosphate buffer saline (PBS, 1x) (Sigma-Aldrich, catalog number: P-4417)

12. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
13. Acetic acid (Sigma-Aldrich, catalog number: 320099)
14. Methanol (Sigma-Aldrich, catalog number: 34860)
15. Acridine orange (Sigma-Aldrich, catalog number: A8097)
16. 70% ethanol
17. Metallodrugs (at IC<sub>50</sub> values)
18. FBS

B. *Allium cepa* assay

1. Onions bulbs (*Allium cepa*), approximately 1.5-2.0 cm in diameter
2. Razor blades
3. Pipette tips
4. Glass microscope slide (75 x 25 mm) (VWR, catalog number: 48300-025)
5. Coverslips (Fisher Scientific, catalog number: 12-543D)
6. Eppendorf tubes 1.5 ml (VWR, catalog number: 89000-028)
7. Test tubes (10 ml) (BOROSIL, catalog number: 9820U04)
8. Pencil with an eraser
9. Nail polish (nitrocellulose dissolved in butyl and ethyl acetate)
10. Double distilled water (ddH<sub>2</sub>O), standard for highly purified laboratory water for biochemical experiments.
11. Hydrochloric acid (e.g., Merck, catalog number: HX0603-75)
12. Acetic acid (e.g., Merck, catalog number: AX0073-6)
13. Schiff's reagents (HiMedia Laboratories, catalog number: S074-500ML)
14. Nail polish

**Equipment**

A. Micronucleus assay

1. Laminar flow hood
2. Hemocytometer
3. Pipettes (any pipettes will do)
4. Incubator (any incubator is fine if it maintains 5% CO<sub>2</sub> and moisture)
5. Fluorescent microscope (Nikon Instruments, model: Eclipse E800) with highly sensitive camera (ANDOR, model: Zyla 5.5) controlled by Nikon NIS-Elements software

B. *Allium cepa* assay

1. Incubator

*Note: Any incubator is fine if it maintains 25 °C and 37 °C, 50-60% humidity and 12 h day lighting/ 12 h dark for 48 h.*

2. Light microscope (Olympus, 10x objective)
3. Digital camera (Nikon, model: COOLPIX P100, 10.3 megapixel, 26x zoom)

## **Software**

1. For both Micronucleus assay and *Allium cepa* assays: Microsoft Office Excel

## **Procedure**

### **A. Micronucleus assay**

1. Cell preparation
  - a. Culture the cells according to their requirement (e.g., MRC-5, HCEC, HaCaT cell lines, etc.).
  - b. Remove medium, and then twice rinse cells with 10 ml PBS, removing the PBS after each washing. The PBS should be autoclaved before use.
  - c. Add 1 ml 0.25% trypsin to the cells and incubate at 37 °C for 1-5 min until the cells appear round.
  - d. Add 10 ml culture medium with 10% FBS, and detach the cells by pipetting.
  - e. Count the cells using a hemocytometer.  
*Note: A relatively accurate number of cells each time is crucial.*
  - f. Prepare desired seeding concentration, and then seed cell into dishes or 6-well plates.

2. Growing cells on cover glasses

Harvest cells and plate an appropriate number of cells per dish or per well in a 6-well plate, at least in duplicate in a final volume of culture medium 1 ml. The number of cells for seeding should be determined by the aggressiveness of the treatment. For example, MRC-5 cells are seeded at a density of  $2 \times 10^4$  cells/well and HCEC cells seeded at a density of  $15 \times 10^4$  cells/well; Incubate cells for 24 h in a CO<sub>2</sub> incubator at 37 °C and allow them to attach to the plate/dish.

- a. Before the plating, place the sterile cover glasses slip flat into each well of a 6-well plate.

*Notes:*

- i. *We usually put one coverglass in one well of the 24-well plate; or no more than 2 coverglasses in one well of the 6-well plate, or no more than 5 cover glasses in a 60 mm dish.*
    - ii. *Pipette up and down or shake the dish to make sure cells are not concentrating in the center of the dish well. Make sure there are no air bubbles between the coverglasses and the tissue culture dish.*
  - b. Treat the cells as necessary with the metallodrugs (at their IC<sub>50</sub> values, against immortalized non-cancer cells).
  - c. Incubate the cells in a CO<sub>2</sub> incubator at 37 °C for 48 h.

3. Fixation and staining

*Note: The solutions in washing fixation or staining steps are gently handled.*

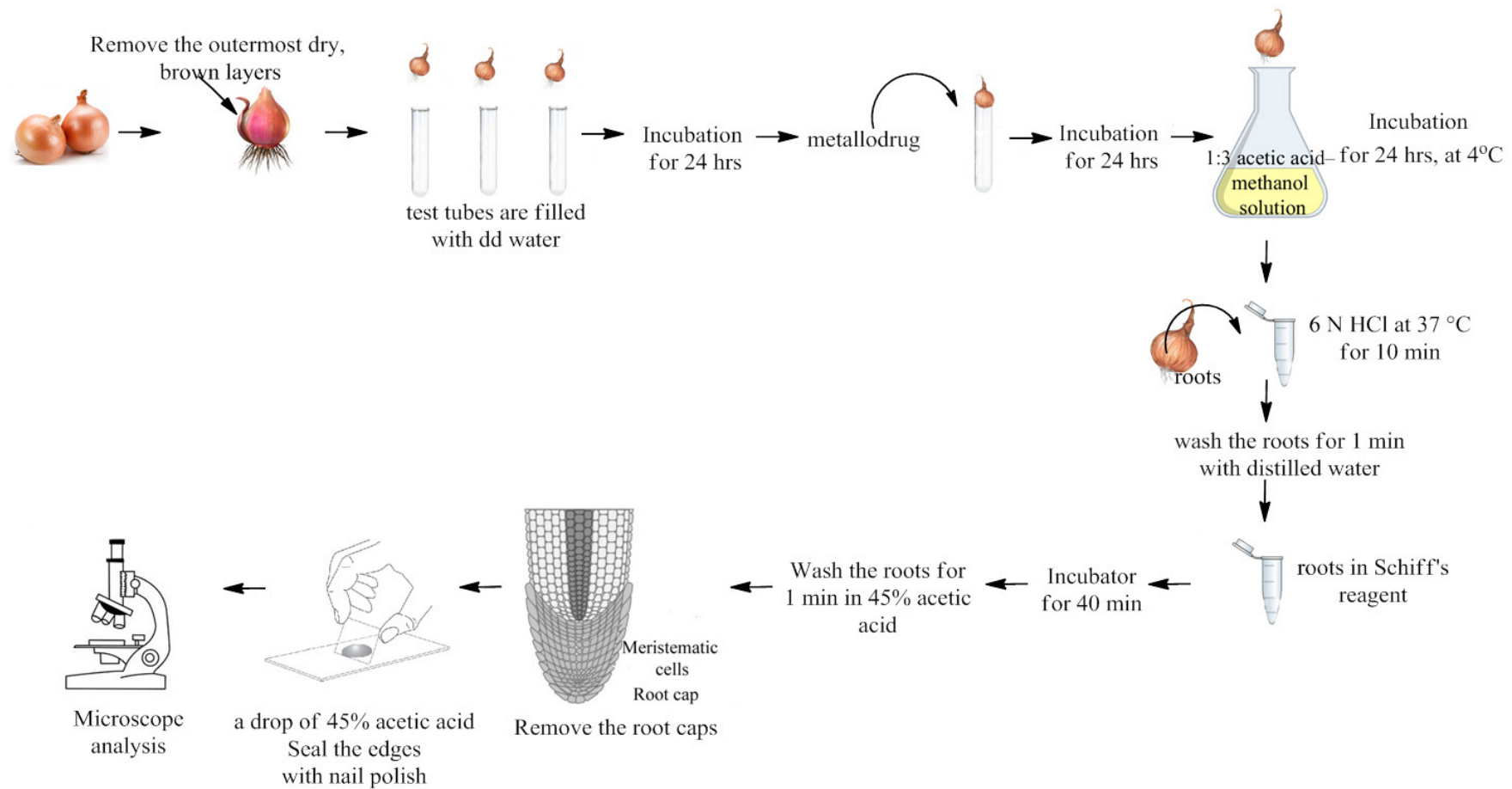
- a. Remove the culture medium after 48 h, and then rinse the cells three times each with 3 ml PBS.
- b. Remove PBS and add 2-3 ml of a hypotonic solution (75 mM KCl) solution and leave the dishes/plates at room temperature (RT) for 10 min.
- c. Remove the KCl, the cells will be fixed by at least three changes of 1/3 acetic acid/methanol with fast rinse procedure. Repeat the procedure three times. Remove the solution of acetic acid/methanol and the cover slips will be also washed with cold methanol containing 1% acetic acid.
- d. Add 3 ml of acridine orange solution (5  $\mu\text{g ml}^{-1}$ ) and incubate for 15 min at 37 °C.
- e. Remove the acridine orange, and the cover slips will be rinsed three times each with 3 ml PBS to remove any excess acridine orange stain.
- f. Get the cover slip from the well and put it face down on the drop of PBS, push it tightly and attach to the glass slide.
- g. The slide now is ready to observe under a fluorescence microscope.

B. *Allium cepa* assay (Figure 1)

1. Purchase fresh onion bulbs (*Allium cepa*, ~1.0-1.5 cm in diameter) from a local grocery store.
2. Remove and discard the outermost dry, brown layers without destroying the root primordia.
3. Place the bulbs of *Allium cepa* on test tubes (10 ml) containing double distilled water.
4. Place the test tubes in an incubator at 25 °C, 50-60% humidity and 12 h day lighting/ 12 h dark for 24 h.
5. Add aliquots from metallodrugs from stock solutions (0.01 M) in the test tubes in order to incubate the bulbs with the metallodrugs, at 3 concentrations, with a range of for example 0.05, 0.5 and 5  $\mu\text{M}$ . The  $\text{IC}_{50}$  value of the metallodrugs towards the cancerous cell lines should be in this range of concentrations.
6. Incubate the *Allium cepa* bulbs (two per dose) with the metallodrugs for 48 h.
7. After 48 h, transfer the roots in tubes and add 5-10 ml 1:3 acetic acid–methanol solution, incubate for 24 h at 4 °C. If the roots are not observed, they can be stored for a few days in 70% ethanol.
8. Transfer the roots into Eppendorf tubes with 1.0-1.5 ml 6 N HCl at 37 °C for 10 min.
9. Remove HCl and wash the roots for 1 min in distilled water.
10. Transfer the root tips into Eppendorf tubes and add 1.0-1.5 ml Schiff's reagent.
11. Incubate the root tips for 40 min at 37 °C.
12. Wash the root tips for 1 min in 45% acetic acid.
13. Remove the root caps from the well stained root tips with a razor blade. The length of roots treated should be about 0.5-2.0 cm.
14. Immerse 1 mm of the meristematic in a drop of 45% acetic acid on a clean microscope slide. Put a coverslip onto mounting solution with one edge contacting the solution first; allow

mounting solution to cover the entire surface of the coverglass, avoiding air bubbles. Squash into single cells using the eraser end of a pencil to apply pressure.

15. Seal the edges of the covering slip with nail polish.
16. The slide now is ready to observe under a fluorescence microscope.



**Figure 1. Experimental steps of *Allium cepa* assay**



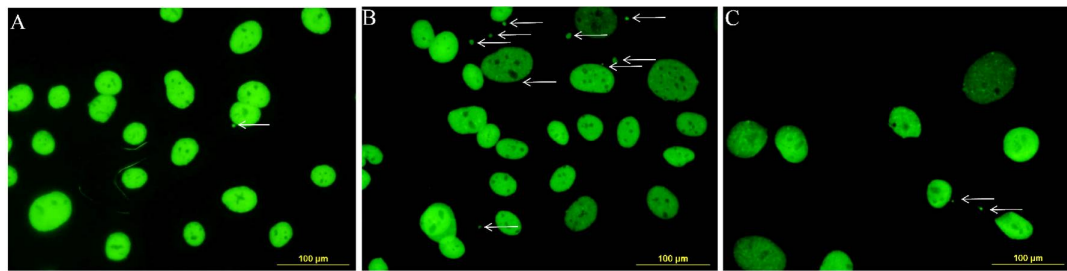
## Data analysis

### A. Micronucleus assay

1. Count the number of micronucleated cells per 1,000 cells.
2. Calculate the percentage (%) of micronucleus as the ratio of micronucleus to the 1,000 number of cells.

*Note: The micronucleus was identified according to the previously established criteria: (1) the area of MN should correspond to 1/256 and 1/9 of the area of the main nuclei, (2) the shape of micronucleus should be round or oval, (3) the micronucleus should be readily distinguished from artifacts, (4) micronucleus is not linked or connected to the main nucleus, (5) micronucleus may touch, but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary and (6) the micronucleus should have the same staining intensity as the main nuclei.*

3. Selected depiction of micronuclei formed in treated with an agent or not HCEC cells is shown in Figure 2.



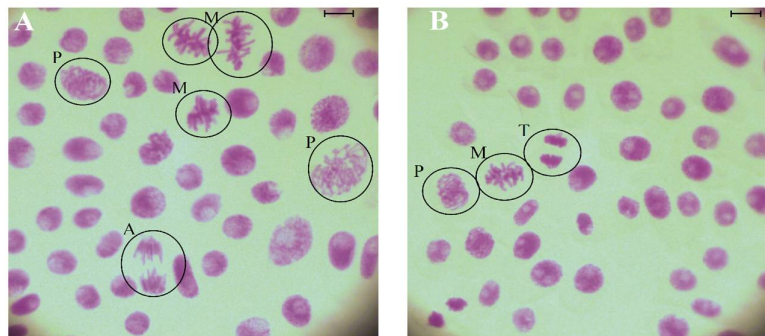
**Figure 2. Selected snapshots of Micronucleus.** Micronucleus which is formed within untreated (A) and treated by ciprofloxacin (B) and CIPAG, a conjugate of ciprofloxacin with silver(I) (C), HCEC cells; arrows indicate micronucleus in HCEC cells. The line bars correspond to 100 μm.

### B. *Allium cepa* assay

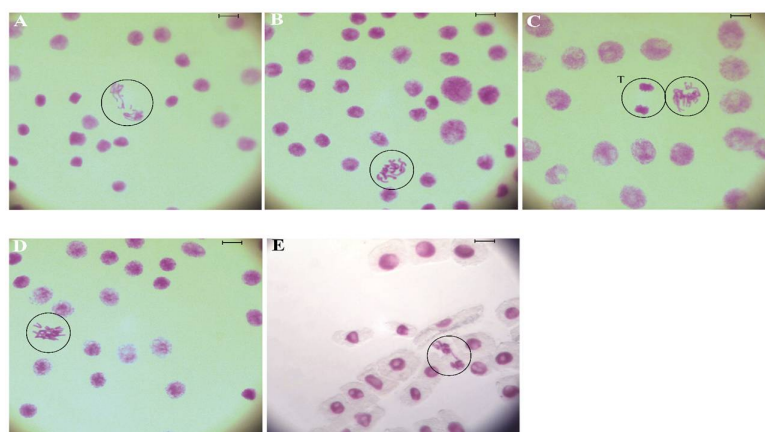
1. The Mitotic index (MI), is the ratio between the total number of dividing cells in cell cycle (prophase, anaphase, metaphase, telophase) to the total number of cells. In order to evaluate the rate of the cellular division the microscopic parameter of the mitotic index (MI) was determined. All categories were analyzed by counting 1,800 cells per concentration (300 cells per slide, total of six slides, 3 slides per onion). The cytotoxicity levels of an agent can be determined by the increase or decrease in the MI.
2. Chromosome aberrations (CA) are observed in the different phases of cell division (prophase, metaphase, anaphase and telophase). CA can be observed as chromosome bridges and breaks, chromosome losses, delays, adherence, multipolarity and C-metaphases.



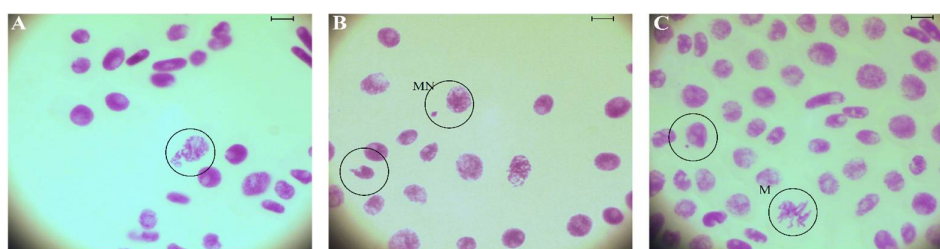
3. Nuclear abnormalities (NA) can be observed in the daughter cells as a similar structure to the main nucleus, but in a reduced size (lobulated nuclei, nuclei carrying nuclear buds, polynuclear cells, mini cells).
4. Micronucleus (MN) can be also observed in daughter cells as a similar structure but in reduced size to the main nucleus.
5. Selected icons of *Allium cepa* meristematic cells in which normal prophase, anaphase, metaphase, telophase, chromosome aberrations, nuclear abnormalities and micronucleus are shown in the Figures 3-5.



**Figure 3. Normal phases of *Allium cepa* meristematic cells.** A. Normal prophase (P), normal metaphase (M), normal anaphase (A) can be observed in *Allium cepa* meristematic cells. B. Normal prophase (P), normal metaphase (M) and normal telophase (T) can be observed in *Allium cepa* meristematic cells. The line bars correspond to 2.5  $\mu$ m.



**Figure 4. Chromosome aberrations observed in *Allium cepa* meristematic cells exposed to chemical agents.** Chromosome loss and bridge at anaphase (A), C-metaphase (B), Chromosome adherence at metaphase (C), Chromosome loss at metaphase (D), Chromosome bridge at telophase (E). The line bars correspond to 2.5  $\mu$ m.



**Figure 5. Nuclear abnormalities and micronucleus are observed in *Allium cepa* meristematic cells which are exposed to chemical agents.** Prophase with synchronic nuclear bud (A), Interphasic nucleus with nuclear bud and Micronucleus in prophase (B), Micronucleated cell in interphase and normal metaphase (M) (C). The line bars correspond to 2.5  $\mu\text{m}$ .

## Acknowledgments

The protocol for Micronucleus assay was adapted and modified from a previous study (Matsushima *et al.*, 1999; Sohaebuddin and Tang, 2013). It was described in Banti *et al.*, 2016; Sainis *et al.*, 2016; Banti *et al.*, 2018; Chrysouli *et al.*, 2018a and 2018b; Latsis *et al.*, 2018; Milionis *et al.*, 2018; Polychronis *et al.*, 2019; Banti *et al.*, 2019.

The protocol for *Allium cepa* assay was adapted and modified from a previous study (Fiskesjó, 1988, Fiskesjó, 1993 and Fiskesjó, 1994). It was described in (Sainis *et al.*, 2016; Chrysouli *et al.*, 2018a and 2018b; Milionis *et al.*, 2018; Stathopoulou *et al.*, 2018).

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## Competing interests

The authors declare no conflicts of interest or competing interests.

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