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Dose-response characterization of *Allium cepa* meristematic cells exposed to 6 MV X-rays

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ABSTRACT

This study investigates the cytogenetic response of *Allium cepa* meristematic cells to 6 MV X-ray irradiation across a wide dose range. The model is recognized as a sensitive and reproducible experimental system for detecting radiation-induced damage. Genotoxic and proliferative effects were evaluated using the micronucleus assay and mitotic index. A strong linear relationship between dose and micronucleated cell frequency was observed in the 0-4 Gy range ($r = 0.99$). Beyond this region, a decline in micronucleated cell frequency and mitotic activity was detected, consistent with radiation-induced loss of mitotically competent cells. The overall dose-response is well described by a linear-quadratic model incorporating a cell-killing component. These findings identify a transition between damage accumulation and proliferative inhibition, supporting the potential of *Allium cepa* as a candidate system for biodosimetric applications involving low-LET photon radiation.

1. Introduction

Research and development of reliable biodosimetric systems is essential for our understanding of radiation effects, both at low and high doses. Radiological protection frameworks aim at limiting risks associated with radiation by accurately assessing the biological damage it may cause (ICRP, 2007; ICRP, 2020). A particularly relevant challenge concerns the evaluation of biological effects in low-dose scenarios, where statistically significant assessment of subtle cytogenetic alterations demands high-throughput, reproducible, and ethically sustainable methods (Fenech, 1993, 2000; Brenner et al., 2003; Xavier et al., 2021). The characterization of dose-response relationships in these conditions is crucial for refining radioprotection models, including the linear no-threshold (LNT) hypothesis, which remains under debate (Goodhead, 2006).

Cytogenetic biodosimetry has become a key tool for studying radiation effects on living systems. Among the available assays, the micronucleus (MN) test and mitotic index (MI) analysis stand out for their simplicity, sensitivity, and robustness in detecting genotoxic and cytotoxic responses to radiation (Fenech and Morley, 1986; Vaijapurkar et al., 2001; Ren et al., 2013; Hayashi, 2016). These endpoints provide quantifiable metrics that can be correlated with absorbed dose, making them useful in both retrospective and predictive contexts (IAEA, 2001).

Although these techniques are traditionally applied to mammalian cells, particularly human lymphocytes (Fenech and Morley, 1986; Senthamizhchelvan et al., 2009; Fenech et al., 2011; Ludovici et al., 2021), recent efforts have increasingly explored plant-based models due to their scalability, ease of handling, and alignment with the principles of the 3Rs (Russell and Burch, 1959). These principles encourage the use of alternative systems that minimize or replace animal experimentation while improving methodological reproducibility. In this context, *Allium cepa* provides a practical and ethically compliant platform for cytogenetic biodosimetry, particularly suited for applications requiring rapid, standardized, and resource-efficient evaluations (IAEA, 2001).

Among plant-based systems, *Allium cepa* is widely recognized for its cytogenetic suitability. Its diploid genome ($2n = 16$), low chromosome

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number, and large chromosome size facilitate the identification of structural aberrations (Grant, 1978; Fiskesjö, 1985). *Allium cepa* has also shown a strong correlation with mammalian cell responses to genotoxic agents (Palmieri et al., 2016; Reis et al., 2017), making it an effective surrogate model. Its ease of use, rapid growth, and high mitotic activity further enhance its suitability for high-throughput screening (Bonciu et al., 2018).

The Allium test has been applied to a variety of physical and chemical stressors, including heavy metals, pesticides, and ionizing radiation (Leme and Marin-Morales, 2009; Xavier et al., 2021; Xavier et al., 2023; d; Errico et al., 2023; Butini et al., 2024). Studies investigating the effects of high-LET (e.g., alpha particles) and low-LET radiation (e.g., X-rays, gamma rays) have confirmed the model's responsiveness through dose-dependent variations in MN and MI (Xavier et al., 2021; Xavier et al., 2023; Bolsunovsky et al., 2019; d; Errico et al., 2023; Butini et al., 2024). Despite the widespread clinical and industrial applications of X-rays, the biological response of *Allium cepa* across a broad range of absorbed doses has not yet been comprehensively investigated.

High-energy photons, such as 6 MV X-rays, induce single- and double-strand DNA breaks, micronucleus formation, and chromosomal aberrations in both cellular and animal models. Human lymphocyte exposure to 6 MV X-rays demonstrates a dose-dependent increase in micronucleated cell frequency, consistent with linear-quadratic dose-response models (Fenech, 2000; IAEA, 2011). At the molecular level, damage arises from both direct ionization by secondary electrons generated through photoelectric and Compton interactions, and from free radicals produced via the indirect effect. Clinically and experimentally, LINAC-generated photon beams (6-15 MV) yield cytogenetic damage profiles comparable to those from ^{60}Co gamma radiation, with microdosimetric differences influencing subcellular damage distribution (0).

However, systematic investigations of the dose-response relationship of *Allium cepa* exposed to high-energy X-rays remain limited, particularly across extended dose ranges where both genotoxic and cytotoxic effects may interact. Understanding these combined effects is essential for interpreting non-linear biological responses and assessing the suitability of the model for quantitative applications. Its use aligns with international recommendations for ecological radioprotection and the protection of non-human biota (ICRP, 2020), supporting both basic research and environmental risk assessment.

The present study provides a systematic characterization of the dose–response relationship of *Allium cepa* meristematic cells exposed to 6 MV X-rays. By integrating genotoxic (micronucleated cell frequency) and proliferative (mitotic index) endpoints, this work identifies a transition between a low-dose regime dominated by damage accumulation and a higher-dose regime characterized by loss of proliferative capacity. The study further evaluates the suitability of a linear-quadratic model incorporating a cell-killing component to describe the observed biological response.

2. Materials and methods

2.1. Germination of Allium cepa seeds

Following established protocols (d'Errico et al., 2023; Barco et al., 2024; Butini et al., 2024), pesticide-free *Allium cepa* seeds of the round Red Baron onion variety were placed in Petri dishes containing two layers of filter paper serving as a growth medium. The seeds were placed, maintaining a distance of about 1 cm between them. The filter paper was then moistened with 4 ml of sterilized water to ensure adequate humidity and support seedling development. The dishes were sealed and incubated at a constant temperature of (25 ± 1) °C for 3 days, until the seedlings reached a length of approximately 4-5 mm.

2.2. Irradiation

The exposures took place at the San Luca Hospital Center in Lucca, where a linear accelerator was used to generate 6 MV X-rays. A calibrated PTW 30013 Farmer-type® ionization chamber was used to measure the dose rate, which was set at 1 Gy/min. The absorbed radiation doses administered were 0.25, 0.5, 1, 2, 4, 8, and 16 Gy.

Irradiations were performed with seeds placed in plastic Petri dishes (Fig. 1a and b). To ensure uniform and reproducible conditions, a custom-made plexiglass contoured build-up cover was used (Fig. 1c). The cover was designed to eliminate air gaps that disrupt electronic equilibrium and to provide appropriate dose build-up.

It incorporated a precisely machined cavity to accommodate the Petri dish, with a 1.5 cm plexiglass layer above the samples. This thickness was selected based on the Percent Depth Dose (PDD) curves for 6 MV X-rays to ensure electronic equilibrium at the sample depth. This configuration established reference irradiation conditions and minimized variations due to air gaps and surface inhomogeneities.

The cover was positioned directly over the dishes during irradiation. To maintain hydration and further improve dose uniformity, 20 mL of distilled water were added to each dish.

Following irradiation, the seedlings were returned to the incubator for 24 h. They were then submerged in Carnoy's fixative solution (a 3:1 mixture of absolute ethanol and acetic acid) for an additional 20-24 h. Finally, the samples were transferred to 70% ethanol and stored in a refrigerator, as described by Guerra and de Souza (2002).

2.3. Sample preparation

Seedlings were hydrolyzed in 1 M HCl at (60 ± 2) °C for 5 min, rinsed in distilled water, and treated with 45% acetic acid to facilitate cell wall softening. Apical meristems were stained with 2% acetic orcein for 15 min in the dark and then squashed on slides to obtain cell monolayers (Fig. 2).

2.4. Slide compression system

To ensure consistent slide preparation and minimize operator-dependent variability, sample compression was performed using a mechanical device designed to standardize the force applied during cell spreading (Fig. 3). The system consists of a carriage moving under a roller across the microscope slide, applying a controlled and reproducible compression force. Pressure is regulated by a preloaded spring, while an integrated monitoring unit ensures it remains within the optimal range for generating uniform cell monolayers.

This system is an improved version of a previously described device (Butini et al., 2024), with enhanced mechanical stability achieved by redesigning the carriage to maintain continuous roller support and prevent force fluctuations. The slide holder was also modified to allow rotation of the sample between compression passes, promoting homogeneous cell distribution.

For slide preparation, the stained meristematic region was placed on the slide, covered with a coverslip, and subjected to two controlled roller passes with intermediate rotation. This procedure yields reproducible single-cell layers suitable for micronucleus and mitotic index analysis.

2.5. Analysis

Microscopic evaluation was performed using an Olympus Evident BX43 optical microscope at a total magnification of 400 × . For each sample, 1000 cells with intact cytoplasmic membranes were examined. These 1000 cells, observed on a given slide, were used both for the micronucleus assay and for the evaluation of the mitotic index. Four biological replicates were analyzed per dose level, including an untreated negative control, yielding a total of 4000 cells evaluated for each radiation dose.

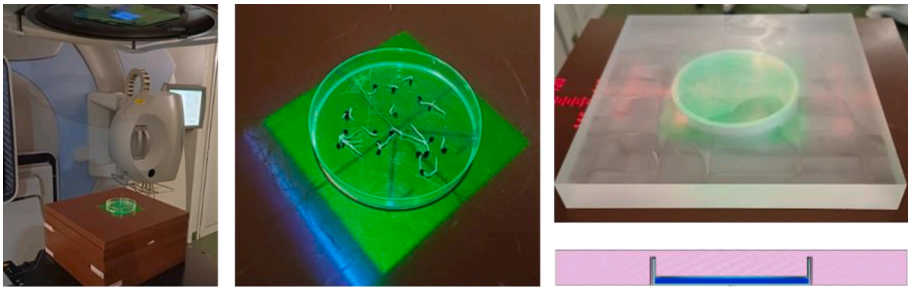

Fig. 1a–c. X-rays irradiation setup using the contoured cover. In the cross section of the cover, the pink area represents the machined plexiglas cover, the green area the Petri dish, and the blue area the water.

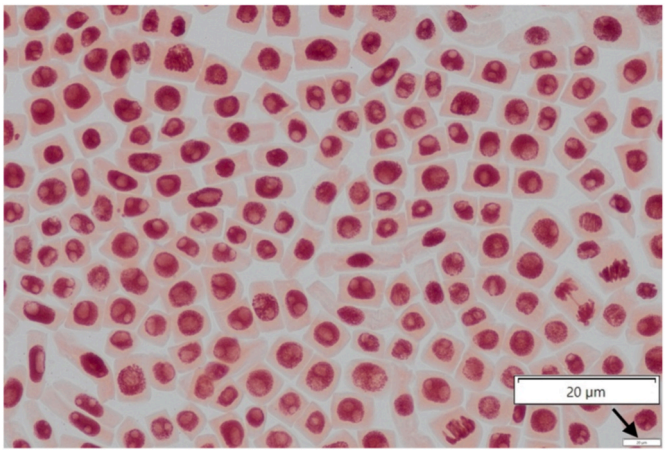

Fig. 2. Example of cells monolayer.

Genotoxic effects of radiation were determined by quantifying the frequency of cells containing one or more micronuclei (Fig. 4), while proliferation was assessed through the calculation of the mitotic index providing indirect insights into cytotoxicity, though it is not a direct measure. A decreased MI may indicate cytotoxic effects, such as cell death or cell cycle arrest, reducing cells entering mitosis. Conversely, an elevated MI could suggest mitotic blockage, as seen with spindle poisons. While MI alone is insufficient for assessing cytotoxicity, it serves as a useful early indicator when paired with cell viability assays.

A double-blind scoring approach was adopted, with two independent investigators evaluating the samples concurrently. A micronucleus was recorded only when both observers reached a consensus. In accordance with the guidelines proposed by Fenech (2000), a structure was classified as a micronucleus only if its diameter ranged between 1/16 and 1/3 of the main nucleus, ensuring consistency in morphological identification.

Cells with metaphase chromosomes are not included for micronucleus scoring because they are in a stage of cell division where chromosomes are fully condensed and aligned, making it impossible to identify micronuclei, which form during later stages like anaphase and telophase. Including them would skew the results, as micronuclei are specifically formed from lagging chromosomes or fragments that are not incorporated into daughter nuclei.

The mitotic index was determined by dividing the number of mitotic figures observed by the total number of cells analyzed (1,000).

Statistical analysis included the Shapiro-Wilk test to verify the normality of data distribution and the calculation of Pearson's linear correlation coefficient. A p-value <0.05 was considered statistically significant. All analyses were performed using the PAST version 4 software (Hammer et al., 2001).

The Shapiro-Wilk test confirmed the normality of the dataset, validating the use of parametric statistics. Consequently, the data are presented in the plots as mean $\pm$ 2 standard deviations (SD), which corresponds to a roughly 95% confidence interval under a normal distribution assumption (Hazra and Gogtay, 2016). This representation was uniformly applied to both the micronucleus counts and the mitotic index, thus facilitating consistent comparison across experimental groups and highlighting any outliers or variability.

Endpoint selection for assessing radiation-induced damage included: the proportion of cells containing at least one micronucleus, the proportion of cells with multiple micronuclei, and the mitotic index. While the mitotic index is not a direct indicator of cytotoxicity, as are viability assays, it can suggest proliferative alterations following irradiation due to cytotoxic stress (Istifli et al., 2019). These parameters align with the standardized micronucleus assay protocols recommended by the International Atomic Energy Agency (IAEA), as outlined in Technical Reports Series No. 405 ("Cytogenetic Analysis for Radiation Dose Assessment") (IAEA, 2001), which endorse evaluating both genotoxic and cytotoxic endpoints for comprehensive biological dosimetry.

3. results

Fig. 5 illustrates the frequency of micronucleated cells in *Allium cepa* seedlings as a function of absorbed dose (0.25, 0.5, 1, 2, 4, 8, and 16 Gy), expressed as the percentage of cells containing one or more micronuclei out of 1000 scored cells.

A progressive increase in micronucleated cell frequency was observed up to 4 Gy, within which the Pearson correlation coefficient was $r = 0.99$ (Fig. 6), indicating an almost perfect linear relationship between administered dose and cytogenetic damage.

This finding is consistent with the results of Butini et al. (2024) for low-dose X-ray exposure and supports the hypothesis that the

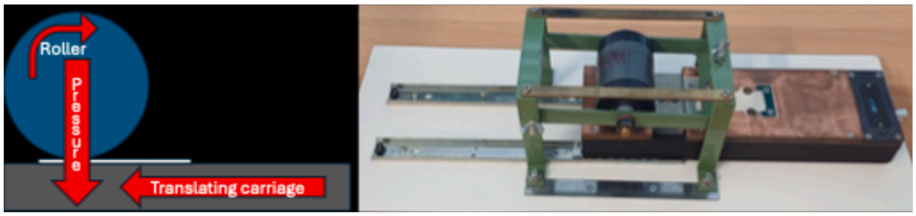

Fig. 3. Compression system.

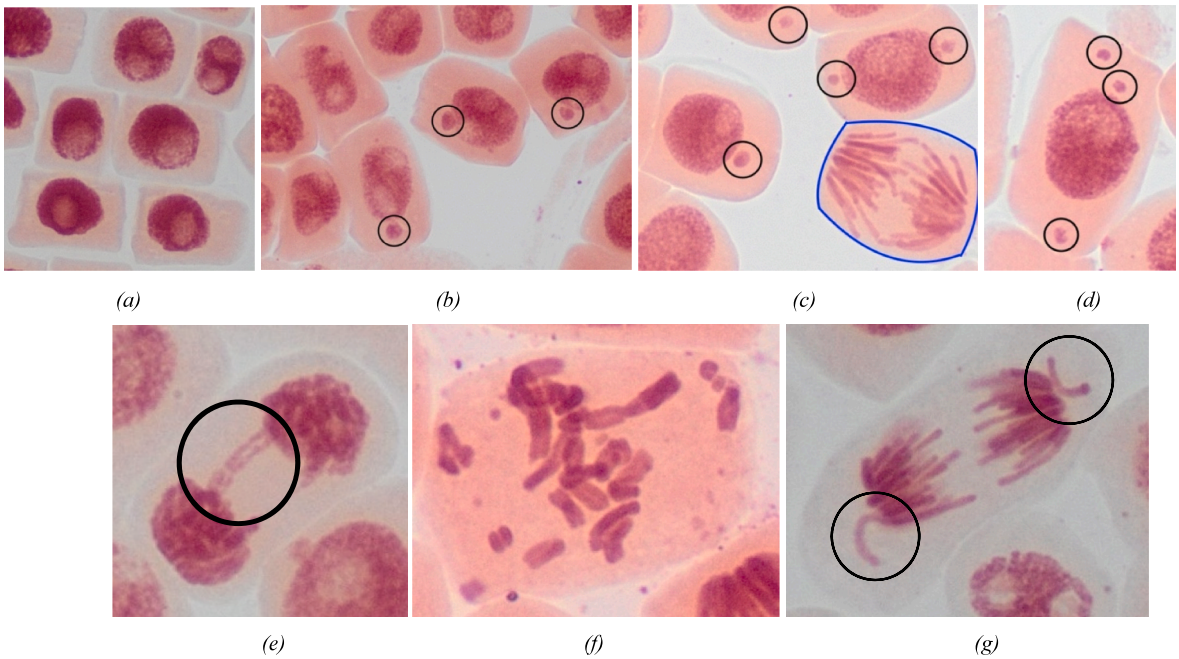

Fig. 4. Representative *Allium cepa* meristematic cells: (a) cells without micronuclei; (b) cells with one micronucleus (black); (c) cells with one or two micronuclei (black) and one dividing cell (blue); (d) cell with three micronuclei (black); (e-g) cells observed after exposure to 8 Gy of X-rays, showing (e) a chromatin bridge, (f) chromosomal damage, and (g) chromosome loss and vagrant chromosomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

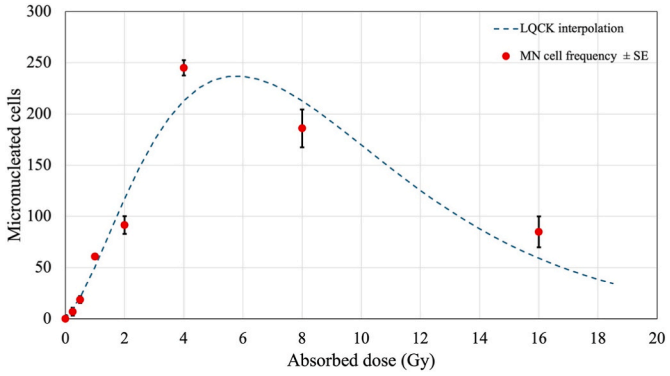

Fig. 5. Linear-quadratic interpolation with cell killing (LQCK) of micronucleated cell frequency as a function of absorbed dose.

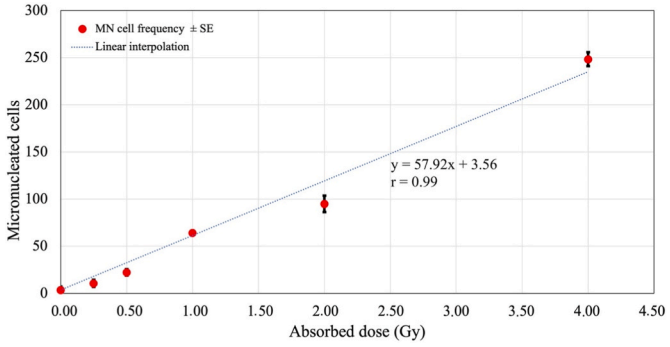

Fig. 6. Linear dose-response trend for micronucleated cell frequency in *Allium cepa* cells between 0 and 4 Gy, with regression line, equation, and Pearson correlation coefficient.

micronucleus assay follows a near-linear possibly, linear-quadratic dose-response relationship at moderate exposure levels.

The maximum frequency of micronucleated cells occurred at 4 Gy, a dose of particular relevance in radiation protection, as it approximates the lethality threshold for humans under acute whole-body exposure in the absence of medical intervention.

Beyond 4 Gy, a reduction in the number of radiation-induced micronuclei was observed. This behavior is consistent with radiation-induced cell death, whereby extensive DNA damage compromises cell viability, preventing the completion of mitosis and the formation of micronuclei. To confirm this interpretation, it will be necessary to complement the micronucleus test with additional assays that evaluate cell viability, such as apoptosis detection or vital dye exclusion tests.

Overall, the dose-response curve appears to follow a linear-quadratic model with a cell-killing component (Fig. 5): at low doses, DNA damage accumulates linearly, while at higher doses, the effect is constrained by a decline in viable, proliferating cells. This type of behavior is widely described in the radiobiological literature and is consistent with the mathematical models used in radiotherapy to characterize tissue radiosensitivity (Brenner, 2008; Santiago et al., 2016).

The frequencies of cells containing one, two, or three micronuclei were also quantified, as shown in Fig. 7. Here as well, a progressive increase was observed up to 4 Gy in the number of polymicronucleated cells (i.e., cells containing multiple micronuclei). This trend reflects the increased complexity of radiation-induced genomic damage, which results in a higher probability of multiple chromosomal breaks and, consequently, multiple micronuclei per cell. Beyond 4 Gy, the number of cells with two or three micronuclei also declined, further supporting the hypothesis of lethal cellular impairment preventing mitotic progression and micronucleus formation.

An additional cytogenetic endpoint was assessed: the mitotic index (MI), representing the percentage of actively dividing cells at each dose point. The results are shown in Fig. 8.

The trend of the mitotic index (Fig. 8) shows a clear dependence on the absorbed dose. Up to 2 Gy, the percentage of dividing cells remains essentially constant, suggesting that in this range, exposure to X-rays does not significantly impair the proliferative capacity of *Allium cepa*

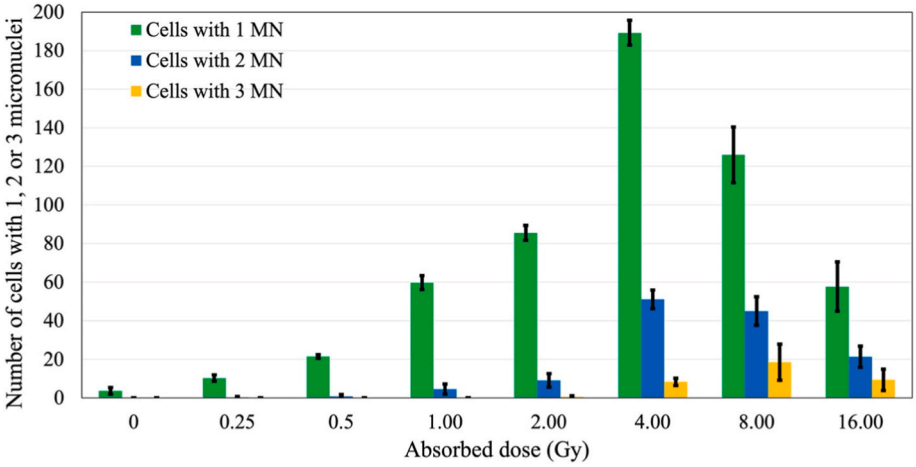

Fig. 7. Number of cells with 1, 2, and 3 micronuclei as a function of dose.

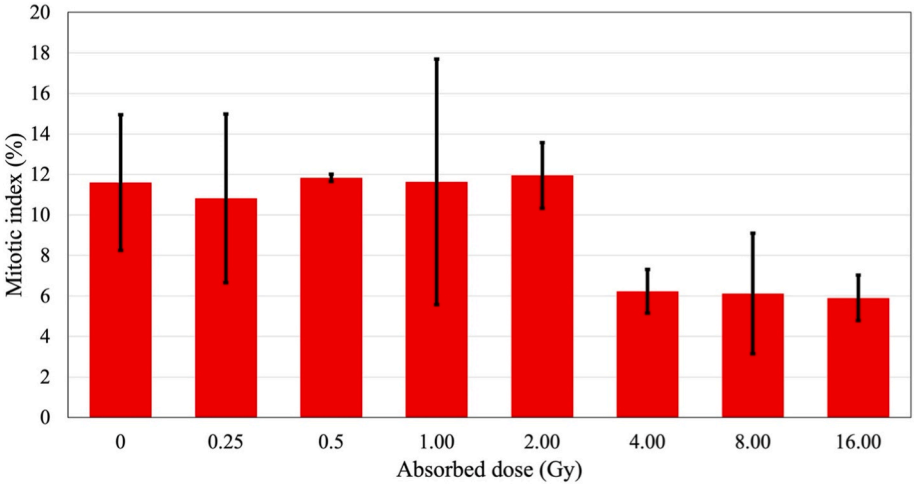

Fig. 8. Mitotic index as a function of dose.

meristematic cells. Starting from 4 Gy, however, a marked reduction in the mitotic index is observed, which stabilizes at lower values and does not show further significant variations with increasing dose up to 16 Gy.

This behavior can be attributed to an increased frequency of irreparable damage to DNA and other essential cellular structures, sufficient to inhibit mitotic progression. The stabilization of the value at higher doses suggests that, beyond a certain threshold, the residual cell population is mainly composed of lethally or sub-lethally damaged cells unable to enter mitosis. This trend is consistent with the analysis of micronucleated cell frequency, where doses above 4 Gy also resulted in a decrease in the number of damaged cells, likely due to the loss of cell viability before the completion of mitosis. To confirm this hypothesis, further experiments will be required, extending the observation period beyond a single cell cycle to assess whether additional cycles influence the balance between damage accumulation and cellular recovery, or further exacerbate proliferative impairment.

4. Discussion

The *Allium cepa* test system is widely recognized for its ability to reproduce key aspects of radiation-induced cytogenetic damage observed in animal tissues, owing to its water-equivalent composition and well-characterized response to genotoxic stress (Grant, 1978; Leme and Marin-Morales, 2009; Palmieri et al., 2016; Reis et al., 2017). Although the onion genome is large and highly repetitive, the presence of conserved orthologous genes with humans (estimated at 15-25%) supports its use as a surrogate model in radioprotection studies. In addition, the use of a structured meristem rather than isolated cells enables investigation of radiation effects within an organized tissue, providing insight into the response of multicellular systems to low-LET radiation (Finkers et al., 2021; Merchant et al., 2007).

The present results demonstrate a well-defined dose-response relationship of *Allium cepa* meristematic cells exposed to 6 MV X-rays, characterized by two distinct regimes. In the low-to-moderate dose range (0-4 Gy), micronucleated cell frequency increased with a strong linear correlation ($r = 0.99$), indicating high sensitivity and reproducibility of the assay for detecting radiation-induced chromosomal damage. This linear behavior is consistent with established cytogenetic biodosimetry approaches and supports the applicability of *Allium cepa* for quantitative dose-response assessment within this range.

At doses above approximately 4 Gy, a deviation from linearity was observed, with a reduction in micronucleated cell frequency despite increasing dose. This behavior is consistent with a decrease in the population of mitotically competent cells due to radiation-induced lethal damage. The overall response is therefore well described by a linear-quadratic (LQ) model incorporating a cell-killing component ($e^{-}\gamma D$), in which damage induction dominates at lower doses, while at higher doses the increasing probability of lethal events limits the expression of cytogenetic endpoints. This interpretation agrees with established radiobiological models describing tissue response to high

doses of low-LET radiation (Brenner, 2008; Santiago et al., 2016).

Additional support for this interpretation is provided by the analysis of polymicronucleated cells and the mitotic index (MI). The increase in cells containing multiple micronuclei up to 4 Gy reflects the increasing complexity of chromosomal damage with dose. In parallel, the MI remained stable up to 2 Gy, indicating preserved proliferative capacity at lower exposures, and decreased markedly from 4 Gy onward, consistent with radiation-induced proliferative inhibition. The concurrent decline in MI and micronucleated cell frequency suggests a reduction in the fraction of cells able to complete mitosis, likely due to lethal or sub-lethal damage. This combined behavior supports the identification of a transition between damage-dominated and cell-killing-dominated response regimes.

Although the MI is not a direct measure of cytotoxicity, it provides an indirect indicator of cell cycle progression by reflecting the proportion of dividing cells. When interpreted alongside genotoxic endpoints such as micronucleated cell frequency, it enables a more robust assessment of the balance between damage accumulation and loss of proliferative capacity. The integration of these endpoints represents a strength of the present approach, particularly for interpreting non-linear dose-response relationships.

Overall, the results identify a biologically relevant transition around 4 Gy separating two distinct response regimes: a low-dose region characterized by accumulation of cytogenetic damage and a higher-dose region dominated by cytotoxic effects and proliferative arrest. The ability of *Allium cepa* to capture both regimes, including the emergence of non-linear behavior at higher doses, highlights its suitability as a model system for investigating radiation-induced cytogenetic effects in a structured biological context.

From a biodosimetric perspective, the strong linear correlation observed in the 0-4 Gy range supports the potential use of *Allium cepa* as a cost-effective and ethically sustainable system for dose estimation following low-LET X-ray exposure. The identification of a transition region around 4 Gy is also relevant for radiological protection, as it marks the onset of significant impairment of proliferative capacity. Further studies incorporating extended observation periods and complementary assays of cell viability or apoptosis will be required to refine the mechanistic interpretation of the high-dose response and to support potential applications in operational biodosimetry.

5. Conclusions

This study demonstrates that *Allium cepa* meristematic cells exhibit a dose-dependent cytogenetic response to 6 MV X-rays characterized by a transition from damage accumulation to proliferative inhibition. The agreement between micronucleated cell frequency and mitotic index highlights the ability of this model to capture both genotoxic and proliferative effects. These findings support the use of *Allium cepa* as a sensitive and biologically informative system for investigating radiation-induced damage and its potential application in biodosimetric research.

CRediT authorship contribution statement

T. Butini: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **F. Barco:** Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **M. Quattrocchi:** Investigation. **R. Ciolini:** Investigation. **M. Marrale:** Supervision. **M.G. Cascone:** Supervision. **E. Rosellini:** Supervision. **M.N. Xavier:** Methodology. **S.O. Souza:** Supervision, Validation, Writing – review & editing. **F. d'Errico:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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