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Glyphosate-based pesticides affect cell cycle regulation

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Abstract

Cell-cycle dysregulation is a hallmark of tumor cells and human cancers. Failure in the cell-cycle checkpoints leads to genomic instability and subsequent development of cancers from the initial affected cell. A worldwide used product Roundup 3plus, based on glyphosate as the active herbicide, was suggested to be of human health concern since it induced cell cycle dysfunction as judged from analysis of the first cell division of sea urchin embryos, a recognized model for cell cycle studies.

Several glyphosate-based pesticides from different manufacturers were assayed in comparison with Roundup 3plus for their ability to interfere with the cell cycle regulation. All the tested products, Amega, Cargly, Cosmic, and Roundup Biovert induced cell cycle dysfunction. The threshold concentration for induction of cell cycle dysfunction was evaluated for each product and suggests high risk by inhalation for people in the vicinity of the pesticide handling sprayed at 500 to 4000 times higher dose than the cell-cycle adverse concentration.

Keywords: Cell cycle regulation; Glyphosate; Glyphosate formulation products; CDK1- cyclin B activation

1. Introduction

Cell cycle is the universal process by which cells reproduce and which underlies the growth and development of all living organisms. Considerable progress have been made on the molecular basis of cell cycle control during the past fifteen years concretized by the 2001 Nobel price for Medecine and Physiology to Lee Hartwell, Paul Nurse and Tim Hunt (Nasmyth, 2001; Dorée, 2001). A considerable body of evidence has proven the highly conserved molecular basis of cell cycle from single unicellular eucaryotes to complex metazoans such as humans and the crucial role played by the protein kinases known as cyclin-dependent kinases (CDKs) in the cell cycle transitions (Nurse, 2000; Nigg, 2001; O'Farrel, 2001; Dorée and Hunt, 2002). Eucaryotic cells have developed control mechanisms known as cell-cycle checkpoints (Hartwell and Weinert, 1989) that restrain cell-cycle transitions in response to stress, allowing the repair of cellular damage or eventually leading to programmed cell death. Cell-cycle dysregulation is a hallmark of tumor cells and human cancers. Failure in the cell-cycle checkpoints leads genomic instability and subsequent development of cancers from the affected cell (Molinari, 2000; Stewart et al., 2003).

Worldwide, there is increasing usage of pesticides in agriculture, industry and for domestic applications that results in a great part of the population exposed to these compounds (Maroni, 2000). The immediate risk of accurate or accidental expose to high levels of pesticides can be readily evaluated, in contrast, the evaluation of risk associated to chronic exposure is a major challenge (Barr et al., 1999; Maroni, 2000). In the field of human cancer, there are correlative or epidemiological lines of evidence that chronic exposure to various environmental products among which pesticides, are associated with increasing frequency of cancers (Barr et al., 1999; De Ross et al., 2003). Since cancers develop years or decades after the primary dysfunction of a single cell (Molinari, 2000), it is a great importance to develop prevention strategies on the basis of the knowledge of the undesirable molecular targets or signaling pathways of pollutants such as pesticides.

Glyphosate is the active herbicide component of Roundup (Malik et al., 1989). Glyphosate alone or with its formulation products was previously considered to be harmless in normal usage and at chronic exposure in previous testing approaches (Williams et al., 2000). However, toxic effect of Roundup at

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sublethal concentration has now been demonstrated in fish (Jraungkoorskul et al., 2003) and other taxonomic groups (Tsui and Chu, 2003). The early development of sea urchin is recognized as a potent model for cell cycle analysis (Nasmyth, 2001; Dorée, 2003) and toxicological studies (Amouroux et al., 1999). Using this model, Roundup was shown to interfere with cell-cycle regulation and thus, was of human health concern (Marc et al., 2002). The molecular terminal target of the glyphosate-based product was the CDK1/ cyclin B complex (Marc et al., 2002 and 2003), the universal regulator of the G2/M transition of the cell cycle (Nurse, 2000; Nigg, 2001; O'Farrel, 2001; Dorée and Hunt, 2002). The pesticide affected the cell division at the level of the molecular switch of the CDK1/cyclin B activation (Marc et al., 2002 and 2003).

Roundup 3plus, the glyphosate-based herbicide, induced cell cycle dysfunction as a result of synergy between glyphosate itself and the mixture of surfactants and permeabilizing agents present in the commercial product (Marc et al., 2002 and 2003). Controversially, the manufacturer claimed that cell cycle dysfunction could have been caused by abnormal formulation of the tested batches or by aberrant concentration of the products (Martens et al., 2002).

In the present article, we show that several glyphosate-based products from different commercial sources and different manufacturers all induce comparable cell cycle dysfunction. The doses-response curves of the formulation products indicate a threshold for induction of cell cycle dysfunction at a concentration much lower than the concentration of the product in the micro-droplets sprayed for herbicide intention suggesting high risk by inhalation for people in the vicinity of spraying.

2. Results

Using the sea urchin early development model, the effect on the cell cycle of several glyphosate-based pesticides was investigated and compared to the already described effect of Roundup using Roundup 3plus (Marc et al., 2002 and 2003). Fertilized sea urchin eggs were incubated in the presence of Amega, Cargly, Cosmic, Roundup Biovert and Roundup 3plus and scored for the occurrence and timing of first cell cycle of sea urchin development. The commercial products contain glyphosate as the common active pesticide ingredient associated with a combination of formulation products. Adverse effects of the different compounds were compared using the concentration of glyphosate as the concentration reference (Fig. 1). Amega, Cargly, Cosmic and Roundup Biovert all impeded the cell division process as reported for Roundup 3Plus (Fig. 1 and Marc et al., 2002 and 2003). The effect was dependent on the concentration of the tested product, ranging from a delay in the timing of the cell division up to a inhibition of the process (Fig. 1). Highly comparable dose-response effects were observed in five to ten independent experiments. The effect of the pesticides was not strictly

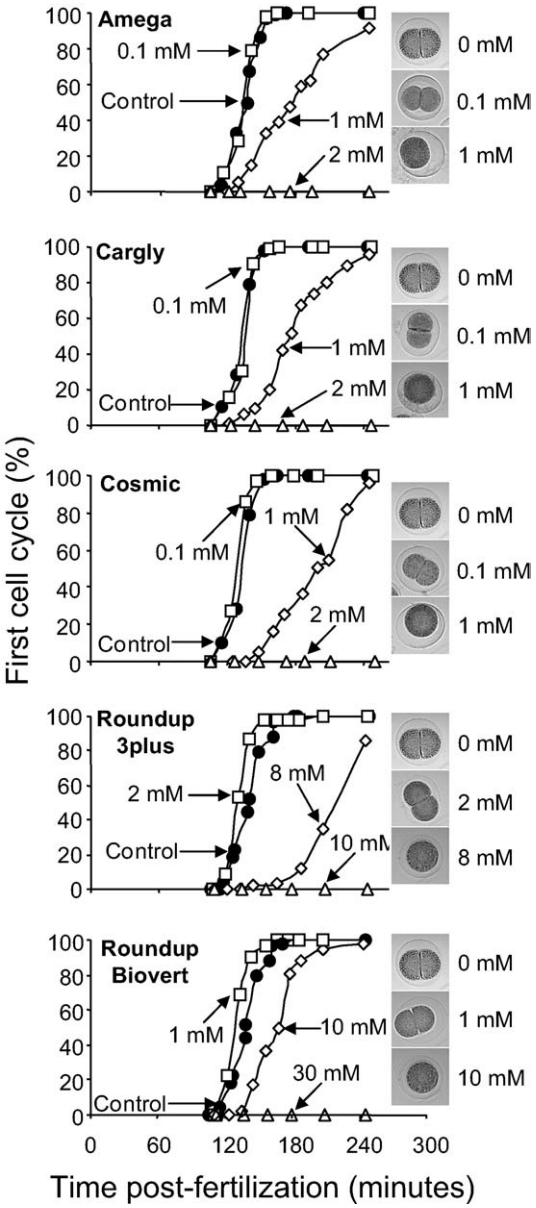

Fig. 1. Effect of glyphosate-based pesticides Amega, Cargly, Cosmic, Roundup 3plus, Roundup Biovert on the kinetic of the first cell cycle of sea urchin development.
Sea urchin eggs were fertilized and the embryos transferred 10 minutes following fertilization into fresh seawater (Control) or into seawater containing formulation products at concentrations indicated in equivalent glyphosate content. First cleavage was scored under phase microscopy observation. The inserts represent control embryos (0 mM) and treated embryos at the indicated equivalent glyphosate concentrations, at 150 minutes post-fertilization. Bar : 30 μm. The figure was obtained from the eggs isolated from a single female and was representative of five to ten independent experiments.

proportional to their content in glyphosate (Fig. 2). Amega, Cosmic and Cargly were consistently found more effective than Roundup Biovert and Roundup 3plus. Half of maximal effect was obtained for glyphosate concentrations around 1 mM in Amega, Cosmic and Cargly compared to 8-12 mM for the Roundup products (Fig. 2).

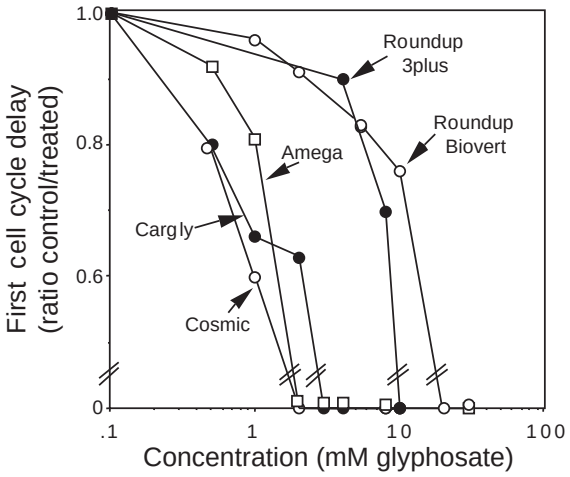

Fig. 2. Dose-response effect of glyphosate-based pesticides Amega, Cargly, Cosmic, Roundup 3plus, Roundup Biovert on the first cell cycle of sea urchin early development.
Sea urchin eggs were fertilized and the embryos transferred 10 minutes following fertilization into fresh water or into seawater containing formulation products at various indicated concentrations, expressed for comparison in equivalent glyphosate content. Kinetic of cell cycle was monitored by phase contrast microscopy observation. Effect of the products on the cell cycle delay is expressed as the ration time for 50 % of the embryo population to reach cleavage stage.

The kinetic of the first cell cycle was affected by each of the products with no necrosis or apoptotic figures as judged from the cytological observations illustrated in inserts of Fig. 1. The morphology of the chromosomes was observed after DNA staining at time intervals after application of the products to the embryos. In all cases, a delay in the morphological changes associated to cell cycle was observed comparable to that reported for Roundup 3plus (Marc et al., 2002 and 2003). However, despite the delay in the cell cycle, no aberrant chromosome morphology was observed during the first cell cycles of early development as illustrated when the control embryos had reached the four cell stage (Fig. 3). Embryos were observed by cytology and for chromosome morphology up to the blastula stage. Comparable results were obtained using any of the five products, and are illustrated with Cosmic (Fig. 4). At a low dose of Cosmic, containing 0.1 mM glyphosate, embryos were not affected in their first cell cycle, and nevertheless were delayed by one round of division, 8th compared to 9th after 24 hours as judged from nucleus counting after DNA staining. At 1 mM glyphosate in Cosmic, the first cell cycles were delayed and the embryos only reached the 3rd cell cycle at 24 hours post-fertilization. When Cosmic was applied at a concentration in glyphosate of 2 mM, the first cell cycle was completely arrested and the embryos progressively turned unhealthy during the 24 hours period.

3. Discussion

Five commercial pesticide formulations containing glyphosate as the active herbicide ingredient induced cell cycle

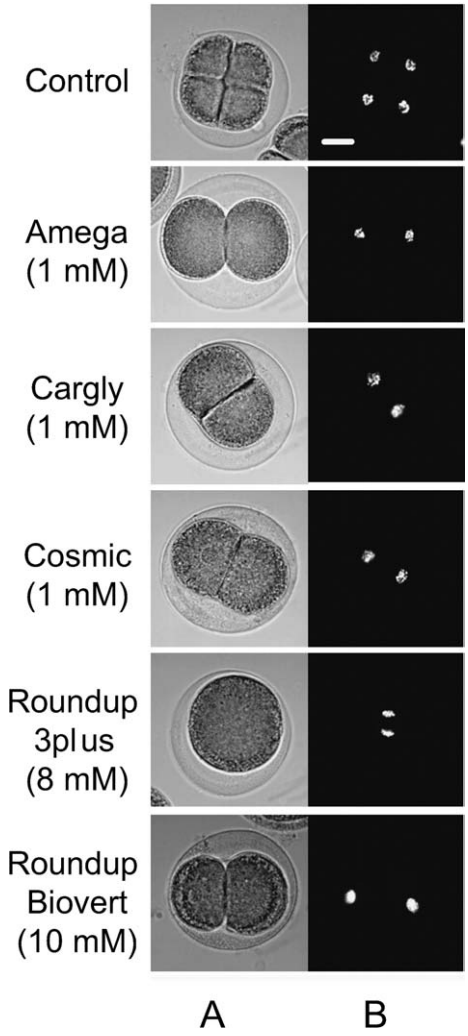

Fig. 3. Glyphosate-based pesticide effect on the time-course of the first cell cycle of sea urchin early development.
Sea urchin eggs were fertilized and the embryos transferred 10 minutes following fertilization into fresh seawater (control) or into seawater containing the glyphosate-based pesticides Amega, Cargly, Cosmic, Roundup 3 plus and Roundup Biovert used at their respective adverse effect concentrations, indicated for comparison in equivalent glyphosate content. At 210 minutes following fertilization, developmental stage and chromatin state were observed respectively by phase contrast microscopy (A) and fluorescence microscopy after DNA staining (B). Bar : 30 μm.

dysfunction as judged from analysis of sea urchin early development. The phenotype of the product effects was comparable to that described and analyzed previously for Roundup (Marc et al., 2002 and 2003) using the Roundup 3plus product. Therefore, the reported effect of Roundup 3plus on CDK1/ cyclin B activation responsible for the delay in the cell cycle appears not to be restricted to a single glyphosate-based product nor to a unique batch of Roundup, but is rather a common feature to the group of products. The effect of Roundup on the whole embryos was ascribed to a synergic contribution of formulation products allowing the cell permeation to glyphosate (Marc et al., 2002). The present results are consistent with such interpretation also in concordance with the synergic action of the formulation product to render glyphosate a potent herbicide. Since gly-

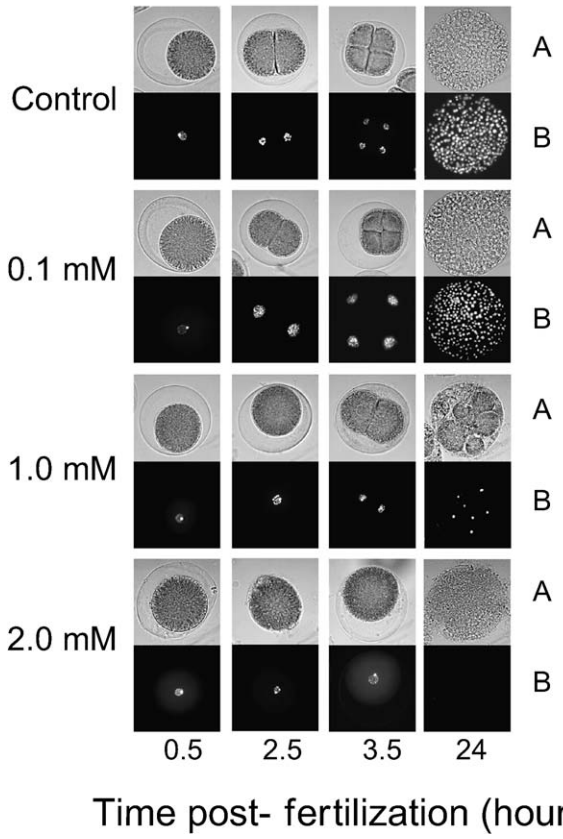

Fig. 4. Cosmic effect on the time course of sea urchin early development. Sea urchin eggs were fertilized and the embryos transferred 10 minutes following fertilization into fresh seawater (Control) or into seawater containing Cosmic at 0.1, 1 et 2 mM equivalent glyphosate. At different times following fertilization, developmental stage and chromatin state were observed respectively by phase contrast microscopy (A) and fluorescence microscopy after DNA staining (B). Bar : 30 µm.

phosate is always spread in combination with the formulation products, adverse effects towards cell cycle regulation must be maximal as long as the formulation products remain together, especially in spayed droplets present in the atmosphere in the vicinity of the pesticide handling. When expressed as their equivalent in glyphosate, the five tested products displayed different efficiencies in inducing cell cycle dysfunction. Cosmic, Amega and Cargly were the most effective whereas Roundup Biovert and Roundup 3plus were less. Since cell cycle disorders such as cancer result from dysfunction of a unique cell (Molinari, 2000; Stewart et al., 2003), it was of interest to evaluate the threshold dose of glyphosate affecting the cells. From the concentration of each product inducing cell cycle disorder around 1 mM for Amega, Cosmic and Cargly and 8-12 mM for the Roundup formulations, the threshold adverse dose of glyphosate sufficient to provoke dysfunction of at least one cell was estimated to be 10 µM when present in Amega, Cosmic and Cargly, and 80-120 µM when present in Roundup Biovert or Roundup 3plus. Since the manufacturers recommend spraying the formulation products at a concentration of glyphosate of 40 mM, the products present in the sprayed droplets are present at 500 to 4000 times higher concentration than the threshold adverse concentration towards the cell cycle. Therefore, glyphosate-based pesticides are clearly of human health concern by inhalation in the vicinity of spraying. Our experiments detect very early a long term risk for humans since cancer may originate from a single cell several years or decades after the initial stress (Molinari, 2000; Stewart et al., 2003). At present, our results demonstrate a molecular link between glyphosate–based products and cell cycle dysregulation, they do not establish a direct link with the development of cancer. However, a recent epidemiologic approach indicates that Roundup may be related to increased frequency of non-Hodgkin's lymphoma among farmers (De Roos et al., 2003) strongly suggesting that early detection of adverse effects using sea urchin early development may be highly useful for in the field of prevention towards chemicals.

4. Material and methods

4.1. Chemicals

Pesticides containing isopropylamine glyphosate salt were from commercial sources: Amega (360 g/l glyphosate) from CFPI Nufarm, Cargly (360 g/l glyphosate) from Cardel, Cosmic (360 g/l glyphosate) from Calliope, Roundup 3plus (170 g/l glyphosate) and Roundup Biovert (360 g/l glyphosate) from Monsanto.

4.2. Handling of eggs and embryos

The sea urchins *Sphaerechinus granularis* were collected in the Brest area (France), kept in seawater and used within 5 days. Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in 0.22 µm-Millipore-filtered seawater, rinsed twice and collected by centrifugation at 2,000 rpm for 2 minutes. For fertilization, eggs were suspended in Millipore-filtered seawater (5 % suspension) containing 0.1 % glycine. Dilute sperm was added to the eggs and withdrawn after fertilization membrane elevation. Experiments were only performed on batches exhibiting greater than 90 % fertilization and each experiment used gametes from a single female. Pesticide solutions were adjusted to pH 7.5 before addition to the embryos suspended in Millipore-filtered seawater. Thousands of embryos were incubated for each experimental determination from which around one hundred were scored for the developmental stage.

4.3. Sea urchin development and cytological observations

Embryos were cultured at 16 °C with constant stirring and observed at short time intervals by phase contrast microscopy for developmental progression. At various times after fertilization, 0.2 ml aliquots of the egg suspension were fixed for at least 2 hours in 0.5 ml methanol/glycerol (3:1) in the presence of the DNA dye bisbenzimide (0.1 µg/ml), mounted in 50 % glycerol and observed under fluorescence microscopy.

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