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Herbicides in Argentina. Comparative Evaluation of the Genotoxic and Cytotoxic Effects on Mammalian Cells Exerted by Auxinic Members

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1. Introduction

In epidemiological and in experimental biology studies, the existence of an increasing interest in biomonitoring markers to achieve both a measurement and an estimation of biologically active/passive exposure to genotoxic pollutants, is nowadays a real fact.
Significant contributions to the advancement of pesticide toxicology came and continue to come from many sources, e.g., academic, governmental/regulatory, and industrial. Regulatory agencies, private sector, and academia worldwide combine expertise to assess pesticide safety and risk potential demanding adequate data of high quality to serve as the basis for establishing safe exposure levels. The extent of testing was and is often determined by the depth of the science, as well as the chemical and physical properties of the agent and the extent of exposure. The importance of pesticide toxicology has evolved from listing poisons to protecting the public from the adverse effects of chemicals, from simply identifying effects (qualitative toxicology), to identifying and quantifying human risks from exposure (quantitative toxicology), and from observing phenomena to experimenting and determining mechanisms of action of pesticide agents and rational management for intoxication. Humans and living species may, therefore, be exposed to a number of different chemicals through dietary and other routes of exposure.
Nonetheless, there continues to be concern that the presence of multiple chemical residues in foods may cause adverse health side effects, including effects that would not be predicted from consideration of single exposures to individual compounds. It is known that the regulatory system for pesticide products found in foods does not routinely address the toxic effects of different substances in combination. The implications, both for risk assessment and for approval processes, of exposure to mixtures of pesticides are among the topics examined by different international agencies, e.g., World Health Organization (WHO, www.who.int), International Agency for Research on Cancer (IARC, www.iarc.fr), United States Environmental Protection Agency (EPA, www.epa.gov), European Chemicals Agency (ECHA, www.echa.auropa.eu), Health Canada Pest Management Regulatory Agency (PMRA, www.pmra-arla.gc.ca), among others. These international agencies, particularly WHO and EPA, have contributed a great deal in their attempts to control pesticide poisoning. They continue their efforts, with particular emphasis on safety in the use of

pesticides and applied research activities, playing the role of intermediary for the involvement of agrochemical industries in safety activities.

It has been strongly recommended that the nature and extent of combined exposure to pesticides and related chemicals, together with the likelihood of any adverse effects that might result, should be evaluated, when carrying out risk assessment. Furthermore, a scientific and systematic framework should be established to decide when it is appropriate to carry out combined risk assessments of exposures to more than one pesticide. Finally, it has been also recommended that groups of pesticides having common targets of toxicological action should be identified (www.food.gov.uk).

Pesticides are ubiquitous on the planet and they are employed to control or eliminate a variety of agricultural and household pests that can damage crops and livestock and reduce the productivity. Despite the many benefits of the use of pesticides in crops field and its significant contribution to the lifestyles we have come to expect, pesticides can also be hazardous if not used appropriately and many of them may represent potential hazards due to the contamination of food, water, and air, which can result in severe health problems not only for humans but also for ecosystems (WHO-FAO, 2009). The actual number of pesticide-related illnesses is unknown, since many poisonings go unreported. It has been estimated that at least three million cases of pesticide poisoning occur worldwide each year (www.who.int). The majority of these poisonings occur in developing countries where less protection against exposure is achieved, knowledge of health risks and safe use is limited or even unknown. Studies in developed countries have demonstrated the annual incidence intoxication in agricultural workers can reach values up to 182 per million and 7.4 per million among full time workers (Calvert et al., 2004) and schoolchildren (Alarcon et al., 2005), respectively. However, the number of poisonings increases dramatically in emerging countries where the marketing of pesticides is often uncontrolled or illicit and the misbranded or unlabelled formulations are sold at open stands (www.who.int). Yet, cases of pesticide intoxication may be the result of various causes in different regions of the world. In emerging countries, where there is insufficient regulation, lack of surveillance systems, less enforcement, lack of training, inadequate or reduced access to information systems, poorly maintained or nonexistent personal protective equipments, and larger agriculturally based populations, the incidences are expected, then, to be higher (IFCS, 2003). Despite the magnitude of the problem of pesticide poisoning, there have been very few detailed studies around the world to identify the risk factors involved with their use. The use of pesticides banned in industrialized countries, in particular, highly toxic pesticides as classified by WHO, EPA, and IARC, obsolete stockpiles and improper storage techniques may provide unique risks in the developing world, where 25% of the global pesticide production is consumed (WHO-FAO, 2009). Particularly, the impact of increased deregulation of agrochemicals in Latin America threatens to increase the incidence of pesticide poisoning, which has already been termed a serious public health problem throughout the continent by the WHO. Many of the pesticides used in Latin America are United States exports and the companies can make a number of changes to ensure the "safe" use of their products. However, the social, economic and cultural conditions under which they are used, pesticides acutely poison hundreds of thousands each year, including many children. In the majority of Latin American countries, poisoning registries are so inadequate that most acute poisoning cases never get recorded. Meanwhile, health effects of chronic or long-term pesticide exposures such as cancer or birth defects are not available, omissions that serve to hide the epidemic proportion of pesticide-related illness in the region. In Argentina, e.g.,

available official data revealed that 79% of the intoxications due to pesticides are related with the use of herbicides followed by insecticides and fungicides (www.msal.gov.ar), values that correlate with the evolution of the phytosanitary market demonstrating that herbicides accounted for the largest portion of total use (69%), followed by insecticides (13%), and fungicides (11%) (www.casafe.org). Consequently, Argentina a larger producer of cereals, including soy, is actually the world eight-largest agrochemical market. The country has seen an explosion in genetically modified soybean production with soy exports topping $16.5 billion in 2008 (www.casafe.org). The fertile South American nation is now the world's third largest producer of soy, trailing behind the United States and Brazil.

2. Herbicides. Auxinic herbicides

The most widely applied agrochemicals around the world are herbicides and consequently the environment is inevitably exposed to these chemicals. Such large amount of herbicides released into the environment may present an impending hazard to living organisms. Exposure to some of these herbicides may lead to alterations in the genetic material thereby causing mutagenicity, carcinogenicity, teratogenicity, and immunotoxicity among other side effects (IARC, 1977, 1999; Dearfield et al., 1999).

The auxinic herbicides have been around since World War II and were the first selective herbicides developed. Herbicides are classified as auxinic based on their growth-promoting effects observed in plant cell cultures, specific tissue systems (coleoptiles, roots), and in whole plants (Pipke et al., 1987; Liu et al., 1999). Generally, the auxinic herbicides are used to selectively control broadleaf weeds in grass crops such as cereal grains and turfgrass swards (Pipke et al., 1987; Reinbothe et al., 1996). These agrochemicals are usually applied as foliar treatments but at higher doses can be used as pre-emergent treatments (Reinbothe et al., 1996). The general susceptibility of dicotyledonous species and tolerance of monocotyledonous species to these herbicides is primarily determined by differences in plant morphology, rate of herbicide translocation and metabolism. For instance, the destruction of the phloem of dicotyledonous species results from abnormal tissue proliferation after exposure to auxinic herbicides. Monocotyledonous species are tolerant since the phloem is scattered in bundles surrounded by protective sclerenchyma tissue. Broadleaf species can be tolerant because they metabolize the herbicide to a less toxic form. These herbicides are considered mimics of the natural plant auxins and are thought to induce changes in gene expression leading to plant death (Reinbothe et al., 1996; Liu et al., 1999). Although they continue to be a very important class of herbicides, their precise mode of action is still unknown. In plants, as it has been stated, these chemicals mimic the action of auxins, hormones that stimulates growth, but in mammals and other species no mimic hormonal activity has been reported (Osterloh et al., 1983).

Among this family of herbicides, the 2,4–dichlorophenoxyacetic acid, commonly known as 2,4-D, and the 3,6-dichloro-2-methoxybenzoic acid, commonly known as Dicamba, are two post-emergent auxinic herbicides released in large amount daily into the environment worldwide. This family of herbicides includes many very effective broadleaf weed killers employed in lawns, golf courses, rights-of-way, and agricultural fields.

2,4-D is an herbicide from the phenoxy acid family that is used post-emergence for selective control of a wide variety of broadleaf and aquatic weeds and forestry applications. It is produced in a variety of forms, including: acid, salt, amine and ester. While at low concentrations 2,4-D acts as an auxin analogue promoting plant growth, increasing cell-wall

plasticity, biosynthesis of proteins and the production of ethylene, at high concentrations it is lethal and is employed as herbicide against broad-leafed and woody plants (Sinton et al., 1986; Devine, 1993; Tripathy et al., 1993). Worldwide, it is the most extensively used herbicide, and third most widely employed in the United States (www.epa.gov).
Dicamba, member of the benzoic acid family, is a chlorinated benzoic acid-derivative compound registered in the United States as a post-emergent herbicide in 1967 (EPA, 1983). It is produced in a variety of forms, including acid and different kinds of salts, e.g., dimethlylammonium salt, potassium salt, and sodium salt, among others (FAO, 2001). This compound is used in different crops, e.g. cereals, maize, sorghum, sugar cane, asparagus, perennial seed grasses, turf, pastures, rangeland, and non-crop land against annual and perennial broad-leaved weeds and brush species (FAO, 2001).

3. Genotoxicity and cytotoxicity of 2,4-D

On the basis of its acute toxicity, 2,4-D has been classified as a class II member (moderately hazardous) by WHO (http://www.who.int/ipcs/publications/pesticides hazard/en/) and slightly to moderately toxic (category II-III) by EPA (EPA, 1974).
Genotoxicity and cytotoxicity studies have been conducted with this auxinic member using several end-points on different cellular systems. A summary of the results reported so far is presented in Table 1. On bacterial systems, either the Ames test or reverse mutation tests performed on both *Salmonella typhimurium* and *Bacillus subtilis* gave negative results regardless of the presence or absence of a rat liver metabolic activation system (Charles et al., 1999; Grabinska-Sota et al., 2002). Whereas the herbicide induced DNA adducts on *Saccharomyces cerevisiae* (Teixeira et al., 2004), negative results were obtained for the induction of unscheduled DNA synthesis in primary rat hepatocytes (Charles et al., 1999). When tested for its carcinogenic potential, the transformation assay in Syrian hamster embryo assay gave positive results (Maire et al., 2007). Induction of DNA single-strand breaks estimated by the alkaline comet assay was evaluated in normal and transformed cells exposed *in vitro* to 2,4-D. González et al. (2005), Maire et al. (2007), and Sandal and Yilmaz (2010) observed an increased frequency of DNA primary lesions in CHO and SHE cells as well as in human lymphocytes. On the other hand, negative results were also revealed when this end-point was assayed on the same cell type by others researchers (Sorensen et al., 2005; Sandal & Yilmaz, 2010). However, Maire and co-workers (2007) showed that 2,4-D was unable to induce DNA fragmentation in SHE cells. Both González et al. (2005) and Soloneski et al. (2007) demonstrated the ability of the herbicide to induce sister-chromatid exchanges (SCEs) in CHO cells and human lymphocytes treated *in vitro*, respectively. An increased frequency of chromosomal aberrations was reported in V79 cells and human lymphocytes-treated *in vitro* in the presence/absence of rat liver metabolic activation system (Pavlica et al., 1991; Zeljezic & Garaj-Vrhovac, 2004) but not when the S9 fraction was absent (Mustonen et al., 1986). Zeljezic and Garaj-Vrhovac (2004) reported the induction of micronuclei in human lymphocytes regardless of the presence or absence of S9 fraction. The induction of alterations in the cell-cycle progression of different cellular systems including plant and V79 cells, human lymphocytes and bovine cells were reported to occur after *in vitro* exposure to 2, 4-D (Basrur et al., 1976; Bayliss, 1977; Pavlica et al., 1991, 2005; Soloneski et al., 2007). However, González and co-workers (2005) were unable to demonstrate such cytotoxic effect in CHO cells. Finally, controversial results were reported for the cell viability assay on yeast and mammalian cells (Sorensen et al., 2004; Teixeira et al., 2004). Similar end-points for both genotoxicity and cytotoxicity were also applied in *in vivo*

End-point System	Concentration[a]	Results	Referencies
In vitro assays			
Ames test			
Salmonella typhimurium + S9	96.1 - 9610 μg/plate	-	Charles et al., 1999
H17 Rec$^+$, M45 Rec$^-$ reverse mutation			
Bacillus subtilis	3x10^{-5} - 90 kg m^{-3}	-	Grabinska-Sota et al., 2002
Transformation assay			
SHE cells	11.5 - 23 μM	+	Maire et al., 2007
DNA adducts			
Saccharomyces cerevisiae	0.45 - 0.65 mM	+	Teixeira et al., 2004
UDS			
Primary rat hepatocytes	2.42 - 96.9 μg/ml	-	Charles et al., 1999
*A*lkaline comet assay			
CHO cells	200 μM - 4 mM	-	Sorensen et al., 2005
CHO cells	2 - 10 μg/ml	+	González et al., 2005
SHE cells	11.5 - 23 μM	+	Maire et al., 2007
Non-smokers HL	1 - 10 μM	-	Sandal & Yilmaz, 2010
Smokers HL	10 μM	+	Sandal & Yilmaz, 2010
DNA fragmentation analysis			
SHE cells	4.5 - 34 μM	-	Maire et al., 2007
SCE assay			
CHO cells	2 - 10 μg/ml	+	González et al., 2005
Non-smokers HL	10 - 50 μg/ml	+	Soloneski et al., 2007
Chromosomal aberrations			
V79 cells	10 μg/ml	+	Pavlica et al., 1991
Non-smokers HL +/- S9	0.4 - 4μg/ml	+	Zeljezic & Garaj-Vrhovac, 2004
Non-smokers HL	0.125 – 0.350 mM	-	Mustonen et al., 1986
Micronuclei assay			
Non-smokers HL +/- S9	0.4 - 4 μg/ml	+	Zeljezic & Garaj-Vrhovac, 2004
Alteration in CCP			
Daucus carota cells	15 - 30 μg/ml	+	Bayliss, 1977
CHO cells	2 - 10 μg/ml	-	González et al., 2005
V79 cells	10 μg/ml	+	Pavlica et al., 1991
Bovine cells	2 - 20 mg/L	+	Basrur et al., 1976
Non-smokers HL	25 - 50 μg/ml	+	Soloneski et al., 2007
Cell viability			
Saccharomyces cerevisiae	0.45 - 0.65 mM	+	Teixeira et al., 2004
CHO cells	100 - 750 μM	-	Sorensen et al., 2004
In vivo assays			
Root tip assay			
Allium cepa	25 - 100 ppm	+	Kumari & Vaidyanath,

End-point System	Concentration[a]	Results	Referencies
Chlorophyll mutation, specific locus			1989
Oryza sativa	25 - 100 ppm	+	Kumari & Vaidyanath, 1989
Wing spot test			
Wing spot and SLRL test	1 - 10 mM	+	Kaya et al., 1999
Wing spot and white-ivory eye	NA	+	Tripathy et al., 1993
spot test	5 mM	+	Graf & Wurler, 1996
Drosophila melanogaster			
TCRG-TCRB recombination			
Mice thymocytes	0-100 mg/Kg/day	-	Knaap et al., 2003
Alkaline comet			
Clarias batrachus erythrocytes	25 - 75 ppm	+	Ateeq et al., 2005
Non-smokers HL*		+	Garaj-Vrhovac & Zeljezic, 2001
SCE assay			
Chick embryo cells	4 mg/embryo	+	Arias, 2003, 2007
Mouse bone marrow and spermatogonial cells	100 - 200 mg/Kg bw	+	Madrigal-Bujaidar et al., 2001
Non-smokers HL*		+	Garaj-Vrhovac & Zeljezic, 2001; Zeljezic & Garaj-Vrhovac, 2002
Chromosomal aberrations			
Allium cepa cells	NA	+	Ateeq et al., 2002a
Shallot root-tip cells	45 - 450 μM	+	Pavlica et al., 1991
Mouse bone marrow cells	NA	+	Venkov et al., 2000
Mouse bone marrow and spermatogonial cells	3.3 - 33 mg/Kg bw	+	Amer & Aly, 2001
Rat bone marrow cells	NA	+	Adhikari & Grover, 1988
Non-smokers HL*		+	Garaj-Vrhovac & Zeljezic, 2001
Hair follicle nuclear aberration			
Mouse bone marrow cells	1/32 LD50	+	Schop et al., 1990
Micronuclei			
Clarias batrachus and *Channa punctatus* erythrocytes	25 - 75 ppm	+	Ateeq et al., 2002b
	25 - 75 ppm	+	Farah et al., 2003. 2006
Mouse bone marrow			
Non-smokers HL*	NA	-	Schop et al., 1990
		+	Garaj-Vrhovac & Zeljezic, 2001
Alteration in CCP			
Allium cepa	NA	+	Ateeq et al., 2002a
Shallot root-tip cells	45 - 450 μM	+	Pavlica et al., 1991

End-point System	Concentration[a]	Results	Referencies
Chick embryos	2 mg/embryo	+	Arias, 2003, 2007
Mouse bone marrow and spermatogonial cells	50 - 200 mg/Kg bw	-	Madrigal-Bujaidar et al., 2001
Mouse bone marrow	NA	+	Venkov et al., 2000
Non-smokers HL*		-	Zeljezic & Garaj-Vrhovac, 2002

Table 1. Evaluation of 2,4-D-induced genotoxicity and cytotoxicity on different target systems. [a], expressed as reported by authors; *, from agricultural workers occupationally exposed to several pesticides, including 2,4-D. UDS, unscheduled DNA synthesis; HL, human lymphocytes; CCP, cell-cycle proliferation; NA, data not available.

systems. 2,4-D has been reported to induce mutations in plants (Kumari & Vaidyanath, 1989) as well as in insects (Tripathy et al., 1993; Graf & Wurler, 1996; Kaya et al., 1999) but not in mice exposed *in vivo* (Knaap et al., 2003). Ateeq and co-workers (2005) reported an increased frequency DNA single-strand breaks in piscine erythrocytes and in the peripheral lymphocytes of a group of agricultural workers occupationally exposed to the herbicide (Garaj-Vrhovac & Zeljezic, 2001). It should be noted that this later positive result could not be totally committed to the 2,4-D but to other pesticides, since the cohort of donors included in the study was exposed to a panel of diverse pesticides. Several reports were able to revealed that 2,4-D increased the frequency of SCEs in chick embryo and mammalian cells (Garaj-Vrhovac & Zeljezic, 2001; Madrigal-Bujaidar et al., 2001; Zeljezic & Garaj-Vrhovac, 2002; Arias, 2003, 2007), and chromosomal aberrations in plants, mouse, rat and human cells, including human lymphocytes from occupationally exposed workers (Adhikari & Grover, 1988; Schop et al., 1990; Pavlica et al., 1991; Venkov et al., 2000; Amer & Aly, 2001; Garaj-Vrhovac & Zeljezic, 2001; Ateeq et al., 2002a). When the micronuclei induction end-point was employed, whereas positive results were found in the piscine system (Ateeq et al., 2002b; Farah et al., 2003, 2006) and human lymphocytes (Garaj-Vrhovac & Zeljezic, 2001), no induction was found in mouse bone marrow cells (Schop et al., 1990). Finally, non-congruent results were reported when the analysis of the cell-cycle progression was used as and end-point for cytotoxicity. Alterations in the progression of the cell-cycle was reported to occur after 2,4-D exposure of plants, chick embryo, and mouse bone marrow cells (Pavlica et al., 1991; Venkov et al., 2000; Ateeq et al., 2002a; Arias, 2003, 2007). However, others authors were unable to revealed such alterations after *in vivo* exposure to the herbicide in bone marrow and spermatogonial mouse cells as well as in non-smokers human lymphocytes (Madrigal-Bujaidar et al., 2001; Zeljezic & Garaj-Vrhovac, 2002).

4. Genotoxicity and Cytotoxicity of Dicamba

Based on its acute toxicity, Dicamba has been classified as a class II member (moderately hazardous) by WHO (http://www.who.int/ipcs/publications/pesticides hazard/en/) and slightly to moderately toxic (category II-III) by EPA (EPA, 1974).

Genotoxicity and cytotoxicity studies have been conducted with this auxinic member using several end-points on different cellular systems. A summary of the results reported so far is presented in Table 2. When mutagenic activity was assessed in bacterial systems with the *Salmonella typhimurium* Ames test either positive or negative results have been reported (Simmon, 1979; Plewa et al., 1984; Kier et al., 1986). Furthermore, similar situation were

observed in *Escherichia coli* and *Bacillus subtilis* when the reverse mutation assay was applied (Simmon, 1979; Leifer et al., 1981; Waters et al., 1981). Whereas the herbicide was unable to induce mitotic recombination in *Saccharomyces cerevisiae* (Zimmermann et al., 1984), negative and positive results were obtained for the induction of unscheduled DNA synthesis in human primary cells regardless of the presence or absence of a rat liver metabolic activation system (Simmon, 1979; Perocco et al., 1990). Induction of DNA single-strand breaks, estimated by the alkaline comet assay, was evaluated in CHO cells exposed *in vitro* to Dicamba. González et al. (2007) demonstrated an increase in the frequency of DNA lesions in this cell line. Similar observations were reported by Sorensen et al. (2004, 2005) on Dicamba-treated CHO cells cultured in the presence of reduced-clay smectites but not when the clay system was not included within the culture protocol. Both González et al. (2006, 2007, 2009) and Perocco et al. (1990) demonstrated the ability of the herbicide to induce SCEs in CHO cells and human lymphocytes with and without S9 fraction treated *in vitro*, respectively. The induction of alterations in the cell-cycle progression of different cellular systems including CHO cells and human lymphocytes were reported to occur after *in vitro* exposure to Dicamba (González et al., 2006, 2007, 2009). Finally, similar results were reported for the cell viability assay in CHO cells (Sorensen et al., 2004; González et al., 2009). In genotoxic and cytotoxic studies *in vivo*, Dicamba was able to induce different types of lesions. It has been reported the ability of the herbicide to give positive results by using the gene mutation and recombination assays when *Arabidopsis thaliana* was used as experimental model (Filkowski et al., 2003). However, both negative and inconclusive results were reported for the sex-linked recessive lethal mutation end-point on Dicamba-exposed *Drosophila melanogaster* (Waters et al., 1981; Lee et al., 1983). Perocco and co-workers (1990) reported an increased frequency of DNA unwinding rate in rat hepatocytes. It has been also reported that the herbicide is able to enhance the frequency of chromosomal aberrations in the root- and hoot-tip cells of barley (*Hordeum vulgare*) and in rat bone marrow cells (Hrelia et al., 1994). On the other hand, no increased frequency of chromosomal rearrangements has been observed in the durum wheat *Triticum turgidum* by Satyavathi and co-workers (2004). Finally, when the micronuclei induction end-point was employed, positive results were reported in *Tradescantia* sp (clone 03) by Mohamed and Ma (1999).

End-point System	Concentration[a]	Results	References
In vitro assays			
Ames test			
Salmonella typhimurium +/- S9	0 - 5000 μg/plate	+	Plewa et al., 1984
		-	Simmon, 1979; Kier et al., 1986
Rec A- reverse mutation			
Bacillus subtilis	0.01 - 5.0 mg/disk	+	Leifer et al., 1981
Pol A reverse mutation			
Escherichia coli	0 - 5000 μg/plate	+	Waters et al., 1981
	0 - 5000 μg/plate	-	Simmon, 1979; Leifer et al., 1981
Mitotic recombination/Gene conversion			

End-point System	Concentration[a]	Results	Referencies
Saccharomyces cerevisiae	0.1 - 5.0 % (w/v)	-	Zimmermann et al., 1984
UDS			
Human diploid fibroblasts +/-S9	0.1 - 3000 μg/ml	-	Simmon, 1979
Non-smokers HL +/- S9			
	0.1 - 0.8 mg/ml	+	Perocco et al., 1990
Alkaline comet			
CHO cells	50 - 500 μg/ml	+	González et al., 2007
CHO cells	10 μM - 10 mM	-	Sorensen et al., 2004, 2005
			Sorensen et al., 2004, 2005
CHO cells + reduced clay	10 μM - 10 mM	+	
SCE assay			
CHO cells	1 - 500 μg/ml	+	González et al., 2007, 2009
			González et al., 2006
Non-smokers HL	200 μg/ml	+	Perocco et al., 1990
Non-smokers HL +/- S9	0.1 - 0.8 mg/ml	+	
Alteration in CCP			
CHO cells	200 - 500 μg/ml	+	González et al., 2007, 2009
			González et al., 2006
Non-smokers HL	100 - 200 μg/ml	+	
Cell viability			
CHO cells	500 μg/ml	+	González et al., 2009
CHO cells	>1000 μM	+	Sorensen et al., 2004
In vivo assays			
A → G/T → G mutation and recombination assay			
Arabidopis thaliana	120 μg/L	+/-	Filkowski et al., 2003
Sex-linked recessive lethal mutations			
Drosophila melanogaster	NA	-	Waters et al., 1981
	3 - 2000 ppm	IN	Lee et al., 1983
DNA unwinding rate			
Rat hepatocytes	NA	+	Perocco et al., 1990
Chromosomal aberrations			
Hordeum vulgare root- and hoot-tip cells, microsporocytes	NA	+	Hrelia et al., 1994
Triticum turgidum	2 mg/L	-	Satyavathi et al., 2004
Rat bone marrow cells	NA	+	Hrelia et al., 1994
Micronuclei assay			
Tradescania sp. Clone 03	50 - 200 mg/L	+	Mohammed & Ma, 1999

Table 2. Evaluation of Dicamba-induced genotoxicity and cytotoxicity on different target systems. [a], expressed as reported by authors; CCP, cell cycle proliferation; NA, data no available; IN, inconclusive results.

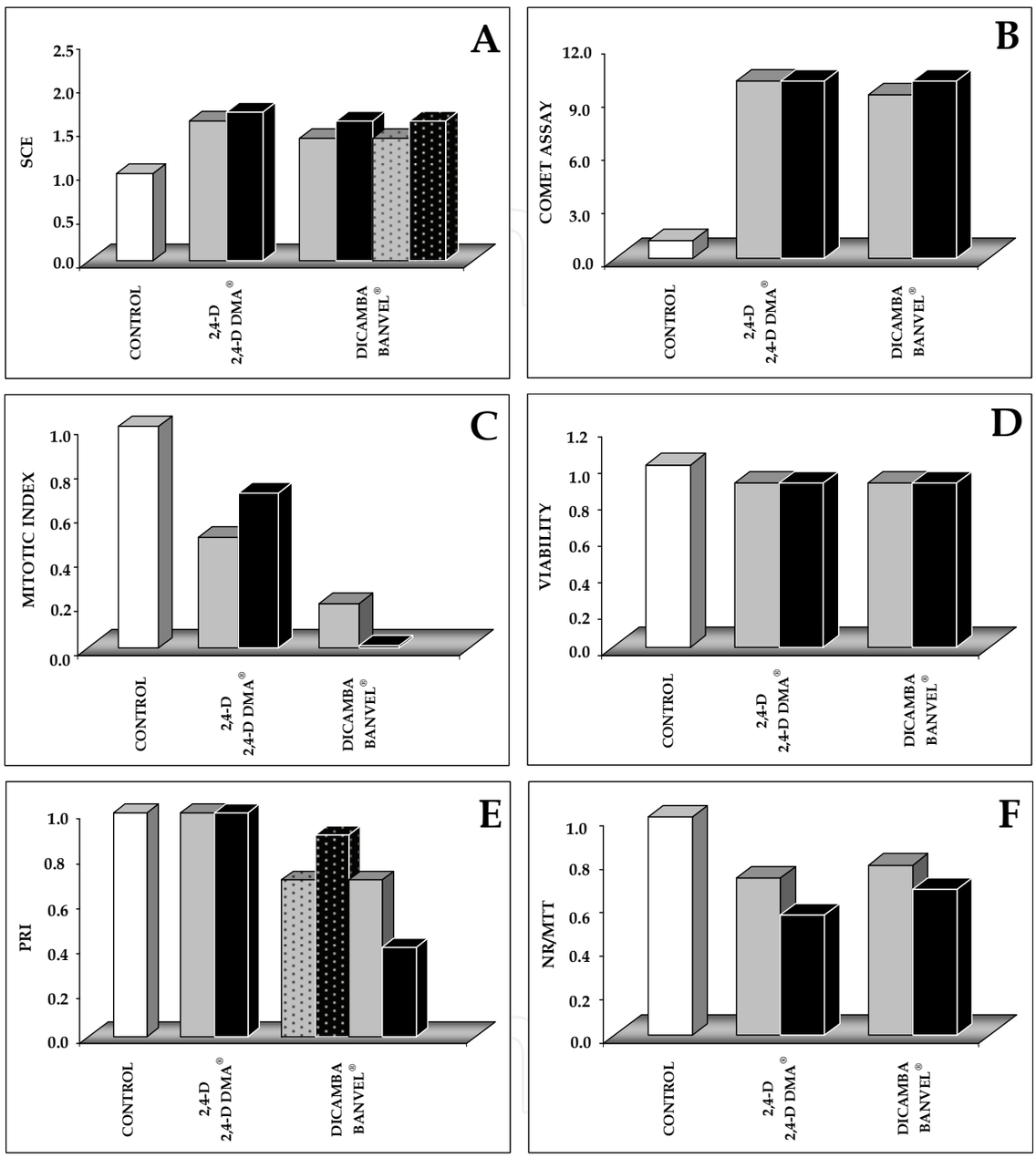

Fig. 1. Comparative genotoxicity and cytotoxicity effects induced by 2,4-D and Dicamba pure herbicides Pestanal® analytical standards (grey bars) and their technical formulations (black bars) commonly used in Argentina on mammalian cells *in vitro* (plain bars, CHO-K1 cells; dotted bars, human lymphocytes). Results are expressed as fold-time values over control data. Evaluation was performed using end-points for genotoxicity [Sister Chromatid Exchanges frequency (A), Comet Assay (B)] and cytotoxicity [Mitotic Index (C), Viability (D), Proliferative Rate Index (E), 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Neutral Red (NR) (F)].

5. Comparison of the genotoxicity and cytotoxicity of 2,4-D and Dicamba and some Argentinean technical formulations

One of the major goals of our research laboratory is to evaluate comparatively the genotoxic and cytotoxic effects exerted by several pure pesticide Pestanal® analytical standards (Riedel-de Haën, Germany) and their technical formulations commonly used in Argentina on mammalian cells *in vitro*. In this section we evaluate comparatively the genotoxic and cytotoxic effects induced in CHO cells and human lymphocytes from non-smoker donors exposed *in vitro* to the auxinic pure herbicides 2,4-D (CAS 94-757) and Dicamba (CAS 1918-00-9) and their technical commercial formulations commonly used in Argentina 2,4-D DMA® (60.2% 2,4-D, Delente Laboratories SRL, Buenos Aires, Argentina) and Banvel® (57.7% Dicamba, Syngenta Agro S.A., Buenos Aires, Argentina), respectively. Evaluation was performed using end-points for genotoxicity [Sister chromatid exchanges frequency and Comet assay] and cytotoxicity [Mitotic index, Cell viability, Proliferative rate index, and 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Neutral Red assays] (González et al., 2005; González et al., 2006, 2007, 2008, 2009; Soloneski et al., 2007).
A summary of the results obtained is presented in Fig. 1. The figure clearly reveals that all compounds assayed are able to inflict DNA damage in CHO cells and human lymphocytes when analyzed at chromosomal and DNA level. We observed that 2,4-D/2,4-D DMA® and Dicamba/Banvel® caused SCEs in mammalian cells indicating that they have clastogenic activity (Fig. 1A). It has been suggested that at the chromosomal level, the induction of SCEs is a reliable indicator for the screening of clastogens, since the bioassay is more sensitive than the analysis of clastogen-induced chromosomal aberrations (Palitti et al., 1982). The results also demonstrate the ability of 2,4-D/2,4-D DMA® and Dicamba/Banvel® to induce DNA single-strand breaks quali- and quantitative analyzed by the comet assay (Fig. 1B). The analysis of the mitotic (Fig. 1C) and the proliferative replication indexes (Fig. 1D) demonstrated that both 2,4-D/2,4-D DMA® and Dicamba/Banvel® are able to exert a marked reduction of the cellular mitotic activity as well as to delay the cell-cycle progression *in vitro* with a concomitant reduction of the proliferative rate index in both cell types. Besides, 2,4-D/2,4-D DMA® and Dicamba/Banvel® are able to induced a clear cellular cytotoxicity, estimated by means of the ethidium bromide/acridine orange assay in CHO cells (Fig 1.E). Finally, a loss of lysosomal activity, indicated by a decrease in the uptake of neutral red, as well as alteration in energy metabolism induced by 2,4-D/2,4-D DMA® and Dicamba/Banvel®, measured by mitochondrial succinic dehydrogenase activity in the MTT assay, were clearly revealed in herbicides-treated CHO cells (Fig. 1F) which corroborate the results obtained applying different end-points for cytotoxicity. Overall, the results clearly demonstrated that the damage induced by the commercial formulations of both herbicides is in general greater than that produced by the pure pesticides, suggesting the presence of deleterious components in the excipients with a toxic additive effect over the pure agrochemicals (Fig. 1). Unfortunately, the identity of the components present within the excipient formulations was not made available by the manufactures. Moreover, though almost improbable, the possibility that the amount of the active ingredient incorporated into the technical Argentinean formulations could be higher than that officially registered cannot be discarded.

6. Final remarks

In agriculture, agrochemicals are generally not used as a single active ingredient but as part of a complex commercial formulation. An active ingredient is a substance that prevents,

kills, or repels a pest or acts as a plant regulator, among others. In addition to the active component, the formulated products contain different solvents, carriers and adjuvants, some of which have been reported to induce damage in mammalian cells, among other cellular systems (Lin & Garry, 2000; Zeljezic et al., 2006; González et al., 2007, 2009; Soloneski et al., 2008; Molinari et al., 2009; Soloneski & Larramendy, 2010). Hence, risk assessment must also consider additional toxic effects caused by the excipient/s. Thus, both the workers as well as non-target organisms are exposed to the simultaneous action of the active ingredient and a variety of other chemical/s contained in the formulated product.

Finally, the results highlight that a complete knowledge of the toxic effect/s of the active ingredient of a pesticide is not enough in biomonitoring studies as well as that agrochemical/s toxic effect/s should be evaluated according to the commercial formulation available in the market. Furthermore, the deleterious effect/s of the excipient/s present within the commercial formulation should be neither discarded nor underestimated.

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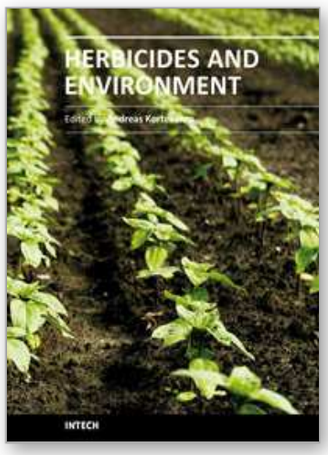

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Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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