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Genotoxic effects of the fungicide thiophanate-methyl on *Podarcis sicula* assessed by micronucleus test, comet assay and chromosome analysis

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Abstract The increasing use of pesticides in modern agriculture has raised the need to evaluate their potential threat to animal and human health. In the present study, the genotoxic effects of environmentally relevant exposure to the fungicide thiophanate-methyl (TM) were assessed in the lizard *Podarcis sicula* (Reptilia, Lacertidae) using micronucleus test, chromosome aberration analysis and single-cell gel electrophoresis (comet) assay. The number of micronuclei increased significantly with exposure time in lizard specimens exposed to 1.5% TM for 30–40 days. In situ hybridization with the specific *Hin*dIII centromeric satellite was positive in 18.7% of the micronuclei observed, suggesting an aneugenic effect of TM during mitosis. DNA damage, evaluated by the comet assay, documented a significant gain in comet length in relation to exposure time that was paralleled by a reduction in head size. Finally, cytogenetic analysis showed a significant increase in chromosome aberrations in exposed animals compared with controls. Our data suggest that long-term TM exposure induces a genomic damage that is positively correlated to exposure time. If such genotoxic effects arise so clearly in an ectothermal vertebrate, such as *P. sicula,* prolonged exposure TM must be considered as a cytogenetic hazard.

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Introduction

The interest in the impact of fungicides is mainly related to their toxicity. Like all pesticides, they can affect human health and the environment, hence the need for assessing their effects (Adams and Moss 2008). Thiophanate-methyl (TM), a thioallophanate compound, is a widespread systemic fungicide with a broader range of action and lower general toxicity compared with other common agents used to control important fungal diseases of crops (Canton 1976; Traina et al. 1998). It is usually sprayed on cereal, vegetable, and fruit crops, on pastures, and on ornamental plants in fields, nurseries and greenhouses. Once absorbed it acts by interfering with microtubule function, impairing tubulin polymerization during cell division, and affecting fungal growth (Fuchs et al. 1972). Ingestion is followed by spread throughout the organism. In humans and other animals TM is metabolized to benzimidazole compounds, including methyl-2-benzimidazole carbamate (carbendazim), through cyclization cleavage of the side chains (Traina et al. 1998; Maranghi et al. 2003).

Data on the toxic effects of TM are conflicting. Whereas high TM concentrations have been seen to induce seizures in rats (Hashimoto et al. 1972) and reversible rashes in rabbits (Noguchi and Hashimoto 1970), a first analysis of bone marrow and spermatogonial cells from rats injected with intraperitoneal TM for 5 days failed to document chromosome structure abnormalities (Makita et al. 1973). In contrast, in vitro studies of human lymphocytes demonstrated increasingly severe chromosomal abnormalities with rising TM concentrations (Hrelia et al. 1996).

According to the U.S. EPA, TM and its derived product, MBC, are associated with low acute toxicity but cause liver and thyroid effects in animal studies, and have therefore been classified as probable human carcinogens. MBC has also been shown to cause adverse effects on the testis. However, dietary exposure to TM residues in food and water is considered to be extremely low, as is the cancer risk posed to the general population.

Reptiles have previously been shown to be excellent indicators of the potential association between contaminants and genetic damage (Hall and Clark Jr 1982; Clark et al. 2000; Talent et al. 2002; Matson et al. 2005, 2009; Sparling et al. 2006; Strunjak-Perovic et al. 2010).

In addition, they bioaccumulate and biomagnify them to levels equal to or greater than those described in birds and mammals (Bryan et al. 1987; Hall and Henry 1992). For this reason, and because of its uniform distribution throughout the Italian peninsula and islands (Bologna et al. 2000), we selected the lacertid lizard *Podarcis sicula* as a biomarker organism to study the effect of TM, introduced in the diet at the concentration normally used by farmers.

Podarcis sicula lives in shady and humid areas, pastures, rich vegetation and, relevantly for our study, on the edges of farmed fields (Bologna et al. 2000).

The genetic and genotoxic effects of TM were assessed by the micronucleus (MN) test, the single-cell gel electrophoresis (SCGE) assay, or comet test, and chromosome analysis. The MN test is a simple and sensitive method to detect both chromosome fragments and whole chromosomes, i.e. both clastogenic and aneugenic activity (Landolt and Kokan 1983; Al-Sabti and Metcalfe 1995). It is often applied in conjunction with the SCGE assay under alkaline conditions (Singh et al. 1988). The latter (pH 13) documents DNA damage. i.e. single-strand breaks and other lesions such as alkali-labile sites, DNA cross-links (Tice 1995; Tice et al. 2000), and incomplete excision repair events (Gedik et al. 1992). Most recent comet assay data come from fish (Buschini et al. 2004; Russo et al. 2004). However, the technique is still being standardized. Finally chromosome analysis was applied to detect and quantify chromosome aberrations such as gaps, deletions and Robertsonian fusion events in metaphase plates.

Comparison of these methods is useful, since it allows estimation of the amount and progression of DNA breakage, which translates into chromosome and/or genome mutation (He et al. 2000).

Materials and methods

Male (M) and female (F) *P. sicula* specimens were captured in the countryside around Naples (Italy) in winter (refractory period) and again in spring (reproductive period). In both cases subjects were taken to the laboratory under controlled conditions and housed in terraria, where they were exposed to seasonal temperature and photoperiod and acclimatized for 20 days, to reverse capture-related stress (Manzo et al. 1994). They were then divided into four groups as follows: Group A, control animals (9 M/9 F) housed in terraria for the whole treatment period and sprayed twice weekly with 100 ml of water; Group B, exposed specimens (10 M/10 F) housed for 15 days in terraria where heather, water and food (larvae) were sprayed twice weekly with 100 ml of 1.5% TM (1.5 g TM in 100 ml of water, the concentration sprayed on fruit crops and ornamental plants); Group C, exposed lizards (10 M/10 F) housed for 30 days in terraria where heather, water and larvae were sprayed twice weekly with 100 ml of 1.5% TM; and Group D, exposed animals (10 M/10 F) housed for 40 days in terraria where heather, water and larvae were sprayed twice weekly with 100 ml of 1.5% TM. The animals were sacrificed at the end of the exposure period. For each treated group 3 M and 3 F of the control group were sacrificed. Tissues were collected and processed for chromosome preparation. Whole blood samples were used for smear analysis and the comet assay.

All efforts were made to avoid animal stress and to minimize the numbers used. The experiments were carried out in compliance with ethical provisions established by the European Union and authorized by the National Committee of the Italian Ministry of Health for in vivo experimentation (Dept. for Veterinary Public Health, Nutrition and Food Safety, D.L. 116/92).

Chromosome aberrations

The number and morphology of chromosomes, obtained from spleen and bone marrow according to In den Bosch et al. (2003), were determined by observing plates stained with 5% Giemsa solution, pH 7, under a Nikon Eclipse E600 fluorescent microscope (Nikon, Tokyo, Japan). The karyotype of *P. sicula* is composed of 38 all acrocentric chromosomes, for this reason abnormalities and rearrangements are easy to be scored (Gorman 1969). The best metaphases were photographed with a CCD camera and analyzed with the CytoVision NT Genus system v3.6 (Applied Imaging, San José, CA, USA) for semiautomatic karyotype reconstruction. About 200 metaphases per group were examined. The results were expressed as the number of each type of aberration per group (Table 1). The chi-square test was used to evaluate the differences in the number and percentage of chromosomal aberrations among groups.

Micronucleus test

Blood was obtained by cardiac puncture using a heparinized capillary tube and smeared on slides. These were fixed

Table 1 Analysis of chromosome aberrations induced by thiophanate-methyl (TM) in *Podarcis sicula*

Days	Number of metaphases examined		Total number of						Total number of aberrations[a]		% TM aberrations	Aberration/ metaphase ± e.s.(σ)	
			Gaps		Breaks		Ch. rearrangements						
	TM	K	TM	K	TM	K	TM	K	TM	K		TM	K
15	197	59	3	0	1	0	0	0	4	0	2.030	0.02 ± 0.01	0
30	201	60	6	0	4	0	1	0	11	0	5.473	0.055 ± 0.02	0
40	190	57	10	2	8	1	3	0	21	3	11.053	0.111 ± 0.02	0.053 ± 0.03

[a] Statistically significant compared with control ($P < 0.05$)

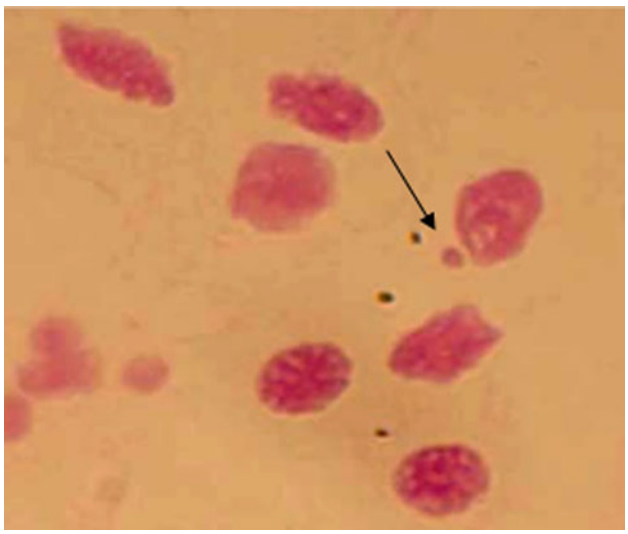

Fig. 1 Micronucleated erythrocyte (*arrow*) of *P. sicula*

in 4% neutral buffered formalin for 30 min prior to Feulgen staining (90 min in Schiff's reagent at RT after acid hydrolysis for 30 min in HCl 2.5 N at 37°C). At least 1000 erythrocytes per specimen were examined under a light microscope. Micronuclei were identified by the absence of connections with the main nucleus and by a nucleus size <1/10–1/30 (Fig. 1). Mean MN frequencies, expressed as the number of micronuclei/1000 erythrocytes, were calculated for each group of animals.

Differences between control and exposed animals were tested using one way analysis of variance (ANOVA) followed by Duncan's test and by Student's *t* test for among groups comparisons. Differences were considered significant when $P < 0.05$.

Fluorescent in situ hybridization (FISH)

A *Hin*dIII centromeric satellite probe of *P. sicula* was used for in situ hybridization. FISH was performed as described by Capriglione et al. (2002).

Alkaline comet assay

The comet assay was performed on *P. sicula* erythrocytes as described by Singh et al. (1988), with some modifications. After cell lysis, DNA was placed in an alkaline electrophoresis buffer for 5 min, followed by denaturation in alkaline solution (Na_2 EDTA 1 mM, NaOH 300 mM, pH 13) and by electrophoresis in the same buffer for 10 min at 25 V, 300 mA. Samples were stained with ethidium bromide (10 μg/ml) and examined under the Nikon E600 fluorescence microscope equipped with a BP 515–560 nm excitation filter and an LP 580 nm barrier filter. Two slides per specimen were prepared and 50 random cells per slide were analyzed. Cells with damaged DNA appeared as comets (Fig. 2), whose tail length was assessed using the CytoVision NT automatic image analysis systemData were analyzed by one-way ANOVA. A *P* value <0.05 was considered significant.

Results

An increased number of micronuclei (Fig. 1) was observed in specimens exposed to 1.5% TM for 30 or 40 days compared with control lizards. The difference was significant and correlated with the length of exposure (Fig. 2). A degree of seasonal variation in MN frequency was also noted, sensitivity to TM being greater in the individuals captured during the reproductive period (spring) (Fig. 2), whose cellular activity is stimulated by higher hormone levels (Andò et al. 1990).

In situ hybridization using the *Hin*dIII centromeric satellite probe demonstrated few (18.7%) positive micronuclei per slide (Fig. 3), suggesting that they may be induced in exposed animals by aneugenic events.

The alkaline SCGE assay documented a significant increase both in the number of damaged nucleotides and in comet tail length in exposed lizards, where the genome damage was significantly ($P < 0.001$) different with respect to control animals (Fig. 5). Like the DNA damage detected by the MN assay (Fig. 3), the genotoxicity

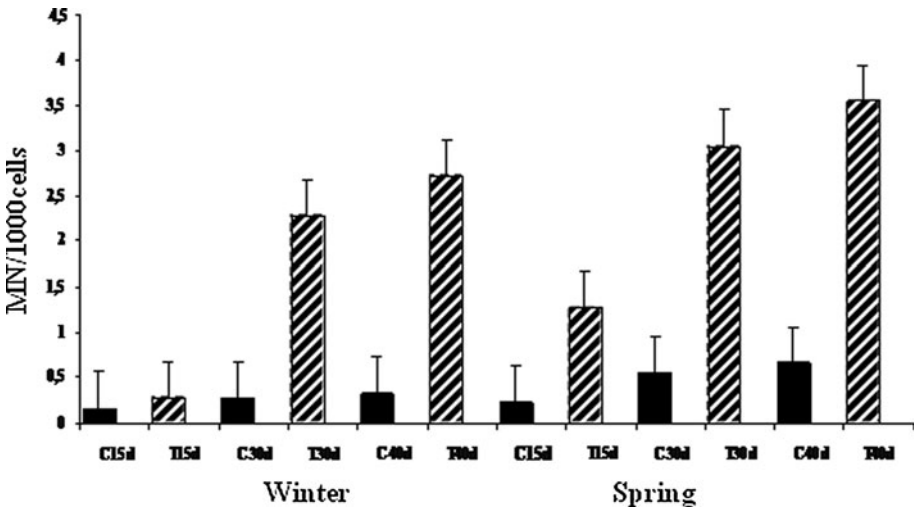

Fig. 2 Mean MN number (micronuclei/1000 erythrocytes). Values are means of counts from 16 individuals, 6 controls (C) and 10 (TM) exposed to TM for 15, 30 or 40 days (d). *Bars* represent standard deviation (SD). Differences between groups are significant when $P < 0.05$

documented by the comet test, i.e. increased tail length paralleled by a reduction in head size (Fig. 4), increased with exposure time.

Cytogenetic analysis of metaphase plates disclosed several chromosome aberrations due to single- or double-strand breaks (Table 1). In Table 1 chromosome aberrations (gaps, breaks and rearrangements) in treated samples are compared with those found in controls (K); the results are expressed as the number of each type of aberration detected in each group. Data show that the overall proportion of aberrations increases with the length of TM exposure. The number of all types of chromosome aberration is significantly ($P < 0.05$) higher in the exposed group than in controls.

Moreover, the majority of gaps appeared to be localized close to the centromere (Fig. 5a).

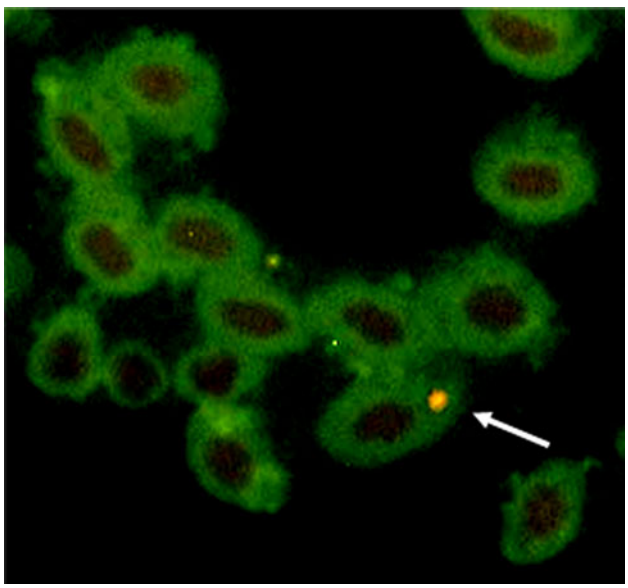

Fig. 3 *P. sicula* micronucleus (*arrow*) exhibiting hybridization with a *Hin*dIII centromere-specific probe (FISH)

Robertsonian fusions (Fig. 5b), easily identified because the chromosomes ($2n = 38$) of *P. sicula* are all acrocentrics, and a reduction in chromosome number were observed in a small number of metaphase plates.

Discussion

This work aimed at adding to the contrasting evidence collected on the genotoxicity of pesticides in general (Bolognesi 2003) and of TM in particular (Hrelia et al. 1996; Saquib et al. 2009).

Both intrinsic and extrinsic factors seem to be involved in the effects of pesticides on genetic material, supporting the value of biomonitoring some marker populations. In this in vivo study we investigated the genotoxic effects of TM, at the concentration normally used in farming, in the lacertid lizard *P. sicula*, because reptiles have been shown as valuable models for ecotoxicological studies and risk assessment both in vivo and in vitro (Talent et al. 2002; Matson et al. 2005, 2009; Sparling et al. 2006; Martinez-Lopez et al. 2010; Strunjak-Perovic et al. 2010).

While early research (Makita et al. 1973; Barale et al. 1993; Traina et al. 1998) tended to underestimate the in vivo and in vitro mutagenic action of TM, recently its genotoxic effect has been recognized to be greater than previously realized (Bolognesi 2003; Saquib et al. 2009). In vivo data obtained through biochemical and histological approaches have clearly demonstrated a time-dependent effect of TM exposure on adrenal and thyroid hormone levels in *P. sicula*, highlighting its ability to affect hormone synthesis and secretion in vivo (De Falco et al. 2007; Sciarrillo et al. 2008). Capaldo et al. (2007) demonstrated that TM also influences the endocrine system response in the amphibian *Triturus carnifex*.

In this in vivo study, exposure of *P. sicula* specimens to TM concentrations similar to those found in their natural

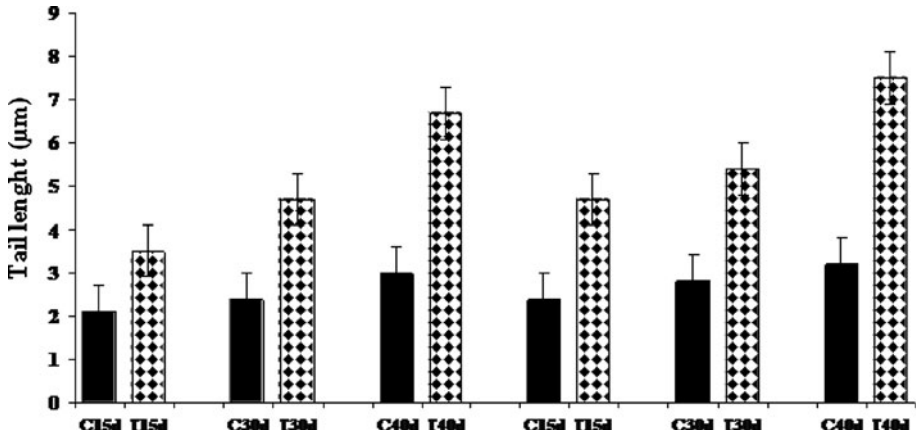

Fig. 4 Mean length of comet tail (in µm). Values are means of counts from 16 individuals, 6 controls (C) and 10 (TM) exposed to TM for 15, 30, or 40 days (d). *Bars* represent standard deviation (SD). Differences between groups are significant when $P < 0.05$

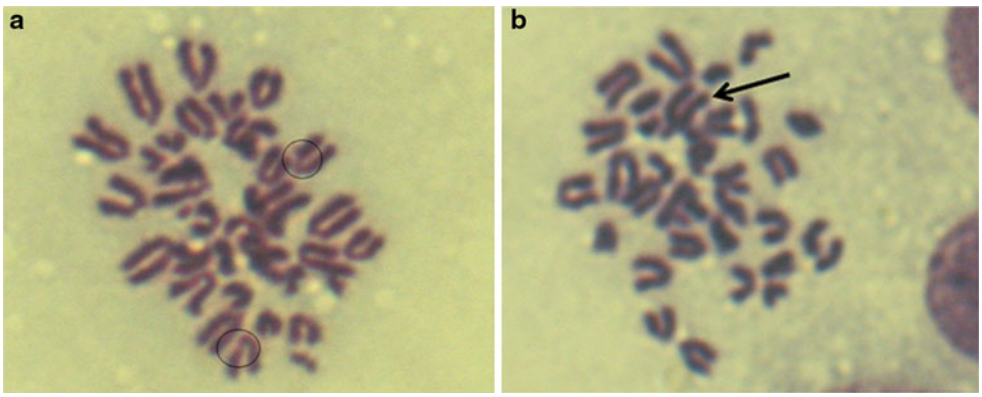

Fig. 5 Giemsa-stained *P. sicula* metaphase plates showing: **a** two chromosomes with percentromeric gaps within the rings, **b** a biarmed chromosome (*black arrow*)

habitats resulted in considerable subcellular damage. All measures of genome damage evaluated, i.e. MN induction, comet tail length, and chromosome abnormalities such as gaps, arm breaks and Robertsonian fusions, were significantly increased in TM-exposed specimens, damage frequency increasing with increasing time of exposure. This probably depends on the fact that TM accumulates in the organism.

A recent in vitro study (Li et al. 2009) has provided valuable insights into the interactions between TM and human serum albumin (HSA), the most abundant plasma transport protein, which has a half-life of 20 days. TM binds tightly to HSA at a high-affinity binding site, altering its molecular conformation (Kirsch-Volders et al. 1997; Li et al. 2009). This may lead to TM accumulation in plasma, enhancing its genotoxic effect.

As regards chromosome aberrations, Hrelia et al. (1996) were the first to provide evidence of the genotoxic effects of TM on cultured human lymphocytes. Here we show for the first time that lizards exposed to TM in vivo had a significantly increased frequency of chromosomal aberrations compared with controls. These findings agree with data from biomonitoring studies of human populations exposed to other contaminants, such as workers involved in pesticide production or individuals living in pesticide-contaminated areas (Sailaja et al. 2006; Ergene et al. 2007). In particular, chromosome aberrations were used as a measure of genome damage, because physical discontinuity and breaks may result in loss of genetic information and/or aneuploidy. Moreover, gaps are held to be linked to chromosomal fragile sites, which are acquired or hereditary regions with relative genomic instability (Yunis and Soreng 1984).

Although the mechanisms underlying gap formation are unclear, multiple factors are known to be involved, including environmental agents such as radiation, cellular response to stress (particularly oxidative stress), and DNA-damaging (chemical) agents (Paquin and Williamson 1984; Bradshaw and McEntee 1989; Licht and Grant 1997). In mammals, these sites are associated with various syndromes and with cancer. In contrast, MN induction and Robertsonian fusion reflect whole or partial chromosome loss or changes in chromosome number, all of which result in karyotype disease.

In our study a proportion of micronuclei hybridized to lizard centromeric satellite DNA. This finding is interesting, because it confirms that TM may induce chromosome

instability by interfering with microtubule formation, resulting in an aneugenic effect. Guerrero et al. (2010) proposed a model of break formation where spindle defects lead to centromere shearing in a carcinogenic cell line with a Dido mutation, which causes spindle defects. They also found loss or gain of complete chromosome arms to be another recurrent genetic defect in carcinoma.

Unfortunately, we detected no sign of aneuploidization, such as cell polyploidization, to confirm this observation.

In conclusion, we demonstrated that TM exposure induced genomic damage (measured as MN induction, comet tail length and chromosome aberrations) and that the frequency of these markers and exposure time are significantly correlated. If such severe genotoxic effects arise in a poikilotherm vertebrate with a low metabolic rate, such as *P. sicula*, prolonged exposure to TM must be considered as a cytogenetic hazard.

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