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Genomic Sensitivity of Small Mammals - A Suitable Test System in the Genetic Monitoring

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1. Introduction

In recent decades, our attention is drawn to human-induced environmental changes and their potential threat to life on Earth through a growing number of mutagens and their effects on the biota. Genetic damage caused by these factors often have a durable effect that is manifested in later generations. The establishment of induced genetic damage in plants, animals and men is one of the most important issues in mutagenesis studies. With each passing year, the environment under anthropogenic influence is loaded with various toxicants. They affect living organisms directly, i.e. on the implementation of individual development, reducing the vitality of the individual, and hence - the population and the species as a whole. Not less important, although more discreetly, their impact remains on the inherited structures of the organisms. It is defined as mutagenic in the broadest sense.

Deleterious alterations in cellular DNA result from endogenous sources of damage as well as from external radiations and genotoxic chemicals in the environment. These alterations can cause mutations, genetic recombination, chromosomal aberrations, oncogenic processes or cell death. All genotoxic agents are capable to induce chromosomal aberrations efficiently (Natarajan, 1993). The types and the frequencies of aberrations induced depend upon the nature of agents, cell cycle stage treated and the cell type employed.

Genetic toxicology deals with the mutagenic effects of chemicals and radiation and reveals their mutagenicity on the biodiversity. Cytogenetic endpoints - chromosomal aberrations, sister chromatid exchanges, mitotic index and micronuclei have long been used to assess exposure of human populations to genotoxic agents. Chromosomal rearrangements are sensitive endpoint towards the action of genotoxins with various origins. Chromosomal aberrations are changes in chromosome structure, which involve gross alteration of the genetic material and are detected using light microscopy. Structural chromosomal aberrations may be induced mainly by direct DNA breakage, by replication on a damaged DNA template, and by inhibition of DNA synthesis (Sorsa et al., 1992; Albertini et al., 2000). They can be divided into two main classes: chromosome-type aberrations involving both chromatids of one or multiple chromosomes and chromatid-type aberrations involving only one of the two chromatids (Albertini et al., 2000; Hagmar et al., 2004). The application of cytogenetic observations is

useful and informative approach in carefully controlled study designs concerning the risk assessment (Sorsa et al., 1990; Vanjo & Sorsa, 1991; Testa et al., 2002).

Cytogenetics offers a direct connection between mutagenicity tests in experimental organisms and effects in humans and is most often used in detecting human mutagen exposures (Bender et al., 1988; Sorsa et al., 1992). Chromosomal aberrations including chromatid breaks, chromatid exchanges, acentric fragments, dicentric chromosomes, ring chromosomes and some inversions and translocations can be reported in peripheral lymphocytes in people who have been exposed to mutagens. Increased frequencies of aberrations were found after exposure to ionizing radiation and various chemicals including benzene, cyclophosphamide, nickel containing compounds, vinyl chloride and styrene (Sorsa et al., 1992) and industrial polymetal dust (Topashka & Teodorova, 2010). Micronucleus formation results from breakage of chromatids or chromosomes when an acentric fragment is produced. Micronucleus test substitutes the metaphase analysis in human monitoring (Sorsa et al. 1992). Therefore, assays detecting chromosomal aberration or micronuclei are suitable for detecting genotoxic agents and clastogens (CSGMT, 1992).

Although all gathered evidences clearly showing the adverse effects of pollution on nature many people still remain unconcerned unless their own health is in danger. Human subjects would provide the most reliable information for risk assessment on human health and ecological risk, but this is not an accepted practice for many ethical reasons. Investigations which use suitable animal test systems and answers they could give may be a potential bridge to meet the gap between the animal-based and human-based environmental observations (O'Brien et al., 1993). The use of mammals to assess the human exposure to toxic environmental contaminants is unquestionable. Pet animals could provide valuable information of whether their owners are threatened by the contaminated environment. Reynolds et al (1994) have found evidence that absorption and excretion of the herbicide 2,4-D occurs readily among dogs after application of the herbicide to lawns. This observation was made particularly during the first 2 days after herbicide application. Hayes et al., (1991) showed a positive association between canine malignant lymphoma, a form of cancer in dogs, and the use of the herbicide 2,4-D by their owners on their lawns and by literature data suggested an increased risk of non-Hodgkin's lymphoma in humans, the histology and epidemiology of which are similar to those of canine malignant lymphoma. Gaines and Kimbrough, (1970) cited by Hansen et al., (1971) reported an increase in mortality among rats whose female parents received approximately 50 mg/kg b. w. per day of 2,4-D in the diet for 3 months before mating and throughout gestation and lactation. Sometimes the influence of toxicants on humans in some cases can be quite disguised or delayed. When hunters use lead pellets, ducks and other species can ingest the lead later, also people or predators that eat these birds can be poisoned (Lightfoot & Yeager, 2008; Ferreyra et al., 2009).

Rodents frequently serve as models to evaluate an exposure risk for humans in ecotoxicological investigations (Shore & Rattner, 2001). Free-living wild rodents are suitable for monitoring the environmental pollution and toxicological risk concerning people living in contaminated regions (O'Brien et al., 1993; Damek-Poprawa & Sawicka-Kapusta, 2003). When small mammals are mentioned, it is generally meant species from two orders: Rodentia and Insectivora. They are used as model organisms, because they have a short life span, co-exist in spatially well-defined small areas, exhibit habitat preferences and feeding behaviour (some are herbivores, omnivores or insectivores) and can be easily caught, marked and studied (Barrett & Pelles, 1999; Metcheva et al., 2003).

Chemical pollutants (which may be considered as component of the general environmental pollution) lead to mutagenesis in somatic and germ cells. As a result of their action the relative frequencies of chromosomal aberrations increase. This fact is used in ecotoxicological investigations to indicate an existing pollution in natural populations (Topashka-Ancheva et al., 2003; Veličković, 2004).

Mutagenic activity of the environmental pollutants is examined on the monitor species considering structural and numerical chromosomal aberrations in somatic cells. In previous investigations some authors (Abramsson-Zetterberg et al., 1997; Ieradi et al., 2003; Zima, 1985) showed that chromosome structures of bone marrow cells in *Apodemus flavicollis* and *Apodemus sylvaticus* are more sensitive to the effect of the environmental pollutants than *Clethrionomys glareolus* and *Microtus arvalis*. Cristaldi et al. (1991) showed an increased micronuclei frequency in bank voles collected in ^{137}Cs contaminated sites. De Souza Bueno et al. (2000) showed a difference in chromosome structure sensitivity of two wild rodent species - *Akodon montensis* and *Oryzomys nigripes* selected as bioindicators in relation to the cytogenetic end points analyzed. These authors pointed out that the mitotic index, the frequency of cells with micronuclei in the bone marrow and peripheral blood, and the frequency of cells with chromosomal aberrations in the bone marrow may reveal a difference in susceptibility to clastogenic agents between the wild species investigated.

Kovalova & Glazko (2006) have revealed the species-specific increase of cytogenetic anomaly frequency in bone marrow cells at a high level of radionuclide pollution and species-specific association between various types of cytogenetic anomalies.

Small mammal species differ in their sensitivity to metal pollution: herbivorous voles and mouse species are regarded as more sensitive than shrew species (Shore & Douben, 1994; Sánchez-Chardi et al. 2007).

Relatively little data is available concerning investigations on changes in chromosomal structure in wild small mammals after an acute treatment of a particular genotoxin under strictly controlled laboratory conditions by comparison with the laboratory mice.

This chapter presents our original findings as well as literature data concerning the effect of some genotoxins on the chromosome structures of four widely distributed in Bulgaria small mammal species and the laboratory ICR mice. These investigations are of present interest, because it is not always correct to extrapolate the genotoxicity findings in laboratory animal models to natural populations. The clastogenic effect of Mitomycin C, $Pb(NO_3)_2$, 2,4-dichlorophenoxyacetic acid (2,4-D) and Tetrahydrofuran was studied on bone marrow cells. The chlorophenoxy herbicide 2,4-D, inorganic lead and organic solvent Tetrahydrofuran are important pollutants of anthropogenic origin. They could affect the chromosomal structures in actively proliferating cell populations, e.g. bone marrow cells and spermatogonial and ovogonial cells as well and thus could be harmful to the genetic material and the organism itself.

1.1 Applied genotoxins

Mutagenic agents we use in this experimental design are one well known clastogen Mitomycin C, as well as some relatively widespread pollutants in the environment - $Pb(NO_3)_2$, 2,4-D and Tetrahydrofuran.

1.1.1 Alkylating agent - mitomycin C (Sigma EC No 200-008-6)

As positive control was selected Mitomycin C - clastogenic agent with proven genotoxic effect, damaging DNA matrix via alkylation. Alkylating compounds are the most active chemical mutagens with high frequency of induced chromosomal aberrations. This compound is a standard laboratory mutagen widely applied on different animal cells (Jena & Bhunya, 1995).

Mitomycin C inhibits DNA synthesis *in vivo* and *in vitro*, reacts covalently with DNA, forming cross-links between the complementary strands of DNA, inhibiting DNA replication (Tomasz et al., 1987), suppress the cell division, thus resulting in induction of chromosomal aberrations.

1.1.2 Lead nitrate $Pb(NO_3)_2$ (Merck, Germany)

Inorganic lead is ubiquitous in the environment because of natural origin or by the industry. Lead is known to replace zinc in many enzymes, including those that are important for proper DNA metabolism and thus can cause fatal injury (Mohammed-Brahim et al., 1985). The lead, cadmium, mercury and arsenic are among the main toxic metals. They accumulate in food chains and have a cumulative effect (Cunningham & Saigo, 1997). Heavy metals often have direct physiologically toxic effects and are sometimes permanently stored or incorporated in living tissues (Bokori et al., 1996).

Lead is a metabolic poison and a neurotoxin that binds to essential enzymes and several other cellular components and inactivates them (Cunningham & Saigo, 1997). Toxic effects of lead are seen on hemopoietic, nervous, gastrointestinal and renal systems (Ma, 1996). Lead can induce single-strand DNA breaks, possibly by competing with metal binding sites in DNA (Shaik et al., 2006).

WHO reported for a few studies in rodents treated with lead salts *in vivo,* which shown small increases in the frequency of chromosomal aberrations and micronuclei in bone-marrow cells, but most of the studies showed no increase (IARC, 1987). Dhir et al., (1993) reported that intraperitoneal (i.p.) injection of low doses lead nitrate in *Mus musculus* caused a significant increase in sister chromatid exchanges rate in bone marrow cells of male Swiss albino mice.

Sharma et al. (1985) found that lead acetate introduced i.p. in ICR mice at doses from 50-200 mg/kg body weight in the 13^{th} day of gestation showed significant increase of sister chromatid exchange rate in maternal bone marrow and foetal liver cells. *In vivo* comet assay studies with Swiss Albino male mice treated by oral intubation with 0.7 up to 89.6 mg/kg body weight $Pb(NO_3)_2$, have shown that lead nitrate causes DNA single strand breaks. No clear dose response between the DNA damage and different doses of $Pb(NO_3)_2$ was detected (Devi et al., 2000).

A significant ecotoxicological risk to a wild population of bank voles (*C. glareolus*), associated with high lead tissue concentration has been estimated by Milton et al. (2003). In Algerian mice (*Mus spretus*), exposed to lead a significant increase in the frequency of micronucleated polychromatic bone marrow erythrocytes and decrease in red blood cells, hematocrit and mean corpuscular hemoglobin were found (Marques et al. 2006).

In a number of studies, lead has been recognized as a potential threat to human health (Goyer, 1993; Jin et al., 2006; Patil et al., 2006). Acidic drinks like tomato juice, fruit juice and others can dissolve lead when packed in inappropriate containers (Karaivanova et al., 2008). This may increase the human risk of lead poisoning. Bilban & Jakopin (2005) found that working in a lead-zinc mine under exposure to radon and heavy metals can result in dramatic consequences for the human DNA (induction of structural chromosomal aberrations) and this is probably more strongly influenced by heavy metals than by radon.

At toxic concentrations, lead acetate and lead nitrate can inhibit DNA repair and damage DNA acting as a comutagen and possibly a cocarcinogen (Roy & Rossman, 1992).

1.1.3 2,4-dichlorophenoxyacetic acid (2,4-D) (Merck, Germany)

2,4-D is an alkylchlorophenoxy herbicide and one of the most frequently used herbicides worldwide as growth regulator for a variety of broad-leaf weeds (while sparing grasses) in agricultural, forestry, and aquatic sites. This herbicide is synthetic structural analogue of natural auxin indole-3-il-acetic acid, which plays a crucial role in division, differentiation and elongation of plant cells. Overdose application of synthetic auxins induces disorganized growth and death in susceptible plant species. There are contradictory results in the literature about the toxicity and possible mutagenic effect of the herbicide 2,4-D, but some investigations emphasize on the observed cellular mutations after exposure which can lead to cancer, immunosuppression, reproductive damage and neurotoxicity (Hayes et al., 1991; Amer & Aly, 2001; Charles et al., 1996b; Rosso et al., 2000). Charles et al., (1996a) found no oncogenic effect of 0-300 mg/kg 2,4-D in mice or 0-150 mg/kg in rats, although slightly increase in primary hepatocellular adenomas were observed in female mice, but with lack of dose response. *In vivo* studies found that 2,4-D included chromatid breaks, chromatid fragments, ring chromosomes, dicentric chromosomes, and chromosome fragments in bone marrow cells in rats (Adhikari & Grover, 1988) and increased rates of sister chromatid exchanges were determined in cultured human lymphocytes (Turkula & Jalal, 1985) if applied at lower dosage. Gonzalez et al. (2005) found that doses of 6 ppm and 10 ppm 2,4-D increased sister chromatid exchanges, reduced mitotic index and increased DNA damage in Chinese hamster ovary cells. In studies in mice Charles et al. *(*1999) indicated that 2,4-D clearly demonstrates a lack of cytogenetic damage at any dose level in the bone marrow micronucleus test. These authors also concluded that 2,4-D is not genotoxic in mammalian systems *in vivo*. Holland et al. (2002) found no statistically significant increase in MN frequency after exposure of 2,4-D in human peripheral blood lymphocytes. Injection of about 2.0 mg 2,4-D into fertile 60 g hen eggs was found to be embryotoxic and decreased in some cases the viability of the chicks, while immersion in a five percent solution had only a moderate effect on hatching (Gyrd-Hansen & Dalgaard-Mikkelsen, 1974). Arias (2003) reported a positive linear correlation between the increase of sister chromatid exchanges and doses (0.5, 1, 2 and 4 mg/embryo) in chicken eggs treated with commercial 2,4-D. Despite these contradictory results, reviews of 2,4-D (Sierra Club of Canada, 2005; Bukowska, 2006; NRDC, 2008; Water Hyacinth Control Program, 2009) determined this herbicide as a genotoxic agent.

1.1.4 Tetrahydrofuran (THF) (Merck, Germany)

THF is a synthesized organic compound, which cannot be found in the natural environment (ACGIH, 2001). THF is used as a solvent in the manufacture of adhesives, lacquers, printing

inks, fats, oils, unvulcanized rubber, etc. Its active ingredient furan is released by combustion of organic waste and thermal treatment of food. Furan is toxic and is a proven carcinogen in laboratory animals (Heppner & Schlatter, 2007).

The probable oral lethal dose of THF in humans is 50-500 mg/kg (Gosselin, 1976). Katahira et al. (1982) evaluated LD_{50} as 1900 mg/kg in rats and 2500 mg/kg in mice after acute toxicity of 20% THF in olive oil by i.p. injection. Chhabra et al. (1998) found a clear evidence of carcinogenic activity in female $B6C3F_1$ mice observing increased incidences of hepatocellular neoplasms at the 1800 ppm exposure level, but these authors did not observe carcinogenic effect of THF in female rats and male mice. Inhalation studies with rats and $B6C3F_1$ mice also provided evidence of carcinogenic activity for THF (Gamer et al., 2002).

THF was not mutagenic in *Salmonella*, micronucleus and DEL tests and did not induce sister chromatid exchanges or chromosomal aberrations in ovary cells (Gamer et al., 2002). According to the data of NTP (1998) in male $B6C3F_1$ mice after i.p. injection of THF at doses of up to 2000 mg/kg significant increase in the number of chromosomal aberrations in the bone marrow cells was not observed. Despite that, ACGIH (2010) confirmed that THF is animal carcinogen with unknown relevance to humans.

1.2 Experimental animals

Model species have been selected to meet the following conditions: they are widespread and are situated low in the food chain, have short reproductive cycle and left several generations a year. Mice of the genus *Apodemus* and voles are well-accepted zoomonitors in environmental pollution studies (Metcheva et al., 1996, 2001). They possess well-studied karyotypes with relatively low number of chromosomes and clearly distinguishable chromosomal morphology (Belcheva et al., 1987; Zima, 2004).

The laboratory mice are well known experimental animals, frequently used as controls in laboratory research. They are the most commonly used mammalian model, more common than rats (Gerasimova & Topashka-Ancheva, 2010).

In this investigation, five small mammal species were used:

Apodemus sp. (Kaup, 1829*)* The description of the karyotype of *Apodemus sp.* was made by R. Matthey in 1936, based on material from Eastern Europe (2n=48, NF=48) and later by other authors (Belcheva et al., 1987; Zima & Kral, 1984). X-chromosome is the largest and Y is the smallest it the karyotype. All authosomes are acrocentric.

Clethrionomys glareolus (Schreber, 1780) (2n=56, NF=59) karyotype consists of 26 pairs of acrocentric and one pair of small metacentric autosomes. X-chromosome is large acrocentric, one of the largest in the complement, while Y is small metacentric like the materials from Central Europe (Belcheva et al., 1987).

Microtus (s.str.) *arvalis* (Pallas, 1778) (2n=46, In Bulgaria NFa=80, NF=84) karyotype is identical with the form "*arvalis*", known from Western and Central Europe; most of the chromosome pairs are metacentric (Belcheva et al., 1977).

Mus spicilegus (Petényi, 1882) karyotype is described for the first time by Painter in 1928 (2n=40). All 19 authosome pairs are acrocentric. Y chromosome is very small and an X chromosome is among the largest acrocentrics of the complement (Mitsainas et al., 2009).

This morphology is characteristic for all subspecies of *Mus musculus musculus* (*domesticus*, *musculus*, *castaneus*, and *bactrianus*) as well as the laboratory lines and the closely related species *Mus spretus* and *Mus macedonicus*.

Mus spicilegus was selected, as it is one of the most frequent species in agroecosystems along with voles.

Male and female laboratory white mice ICR (2n=40) (♂♀=53), weighting 20±1,5 g were delivered from the Slivnitza animal breeding house of the Bulgarian Academy of Sciences, Sofia. Animals were kept at standard conditions at temperature 20-22ºC, photoperiod 7am to 7pm, free access to standard animal food for laboratory animals - "Rodents" (produced by Vitaprot-Ltd., Kostinbrod, Bulgaria, according prescription 456-1-12) and water.

All small mammal species originated from several conditionally unpolluted regions in Bulgaria: Rila Natural Park, Vitosha Nature Park, Kresna Gorge and the vicinities of Pleven region. Apodemus sp. (♂♀=80), *Clethrionomys glareolus* (♂♀=44), *Microtus arvalis* (♂♀=61), *Mus spicilegus* (♂♀=71).

The experimental treatment of the wild model species was conducted not earlier than 48 hours after capture and the structural changes in the chromosomes were scored 24 hours after treatment with the genotoxins.

The experiments were conducted according to approved protocols, and in compliance with the requirements of the European Convention for Protection of Vertebrate Animals used for experimental and other Specific Purposes and the current Bulgarian laws and regulations.

2. Cytogenetical analysis

Bone marrow is one of the most convenient tissues for testing the mutagenic effects of environmental factors due to the low frequency of spontaneous chromosomal aberrations, high cell proliferative activity, relatively rapid and simple method of making the preparations. The prevailing forms of chromosomal damage are those of the chromatid type. The cytogenetical method applied is intended to show the sensitivity of chromosomal aberration assay in animal's bone marrow to determine whether certain components of the environment can induce chromosomal aberrations with a frequency that is significantly higher compared with their frequency in the control animals or in animals from unaffected areas.

Mitomycin C (3.5 mg/kg b.w.) (Sigma EC No 200-008-6), Pb(NO3)2 (200 mg/kg b.w.) (Merck, Germany), 2,4-D (3.5 mg/kg b.w.) (Merck, Germany) and THF (3.8 mg/kg b.w.) (Merck, Germany) were injected intraperitoneally (i.p.) only once. Control animals received only 0.9% NaCl solution. All the genotoxins applied were dissolved in 0.9% NaCl solution. The accurate number of males and females used in the experiments for each toxicant applied is presented in relevant tables.

Our previous data (Venkov et al., 2000) on low clastogenic and multiple effect of 2,4-D which is widely used in the treatment of weeds in agroecosystems gave us reasons to expand our experiments. The herbicide 2,4-D (3.5 mg/kg b.w.) was introduced i.p. three consecutive times at intervals of 48 hours (total 96 hours). Bone marrow cell samples for cytogenetical analysis were prepared 24 hours after the third and last injection of the herbicide (total 120 hours after the initial herbicide treatment).

The cytogenetical analysis was mainly performed according to the protocol described by Preston et al. (1987). To accumulate a sufficient number of metaphases in order to obtain metaphase chromosomes suitable for cytogenetic analysis, one hour before bone marrow cell isolation a mitotic inhibitor colchicine – 0.04 mg /g b.w. was injected. Animals were euthanized by diethyl ether, bone marrow cells were flushed from femur and hypotonized in a 0.075 M potassium chloride at 37ºC for 15 min. Thereafter the cells were fixed in cold methanol: glacial acetic acid (3:1), resuspended and dropped on precleaned cold wet slides and air dried. The slides were stained in 5 % Giemsa solution (Sigma Diagnostic). Up to 50 well-scattered metaphase plates were analyzed from each animal using light microscopy (Cetopan Reichert, Austria) x 1000.

The main types of aberrations – breaks, fragments, exchanges (centromer/centromeric fusions, telomere/telomeric fusions) and pericentric inversions were separately scored.

2.1 Statistical analysis

The frequencies of chromosomal aberrations were determined for each animal. The mean ±SD for each group was calculated and the data was statistically evaluated for their significance by analysis of variance using the Student *t* test. Statistical significance is expressed as $p<0.001$; $p<0.01$; $p<0.05$; $p>0.05$ - (not significant).

3. Results and discussion

The chromosome aberration frequencies in the analyzed bone marrow cells of all five small mammal species investigated are presented in Tables 1-4. The values of the metaphases with aberrations in ICR mice control group are 1.0±0.3%, 3.25±0.36% in *M. spicilegus*, 3.43±0.57% in *M. arvalis*, 1.2±0.42% in *C. glareolus* and 2.66±0.4% in *Apodemus sp*. These percentages of aberrant metaphases were in most cases within the range of frequencies, determined in cells of various small mammal species captured in different wild habitats (Topashka et al., 2003).

There was no statistically significant difference in the percentage of chromosomal aberrations for each experimental group between males and females after experimental treatment with all toxicants under present investigation. That allowed combining the results for both sexes and presenting them as mean ± SD%.

3.1 Mitomycin C clastogenicity

The chosen dose of Mitomycin C was administrated i.p. in all experimental animals and the percentages of chromosomal aberrations are presented in Table 1.

In all investigated species the experimental treated groups showed reliably higher percentage of damaged cells in comparison with the untreated controls ($p<0.01$).

The highest percentage of cells with chromosomal aberrations was observed within the treated group of the laboratory mice ICR (38.42±1.88%) followed by its relative species *M. spicilegus* (21.6±1.45%). Significant reduction of the amount of cells with aberrations in bone marrow of the treated *Apodemus sp.* was observed (11.01±0.81%). The lowest values of cells with aberrations appeared in the species of family Cricetidae: *C. glareolus* (10.38±1.57%) and *M. arvalis* (10.72±0.28%). Obviously, ICR mice showed a three-fold higher sensitivity in comparison with the treated *C. glareolus*, *M. arvalis* and *Apodemus sp*. ($p<0.001$).

These results indicate that all wild small mammal species injected i.p. with Mitomycin C showed significantly lower karyotype sensitivity in comparison with the ICR mice ($p \leq 0.01$). The chromosome structure of both treated species of genus *Mus* is more susceptible to the action of the alkylating agent Mitomycin C ($p < 0.05$).

Chromatid breaks and fragments as though chromosomal rearrangements (centromeric fusions (c/c), telomeric fusions (t/t), pericentric inversions and taranslocations) were separately scored. Breaks and fragments significantly prevail over the chromosomal type aberrations (from 74% of all aberrations observed in laboratory mice, 75% in *Apodemus sp.*, 83.3% in *C. glareolus*, 91.7% in *M. spicilegus* up to 96% in *M. arvalis*). The share of breaks and fragments appears to be the highest in *M. spicilegus, M. arvalis* and *C. glareolus* and lowest in ICR mice.

Compound	Time after treatment	Number of metaphases scored	Type of chromosomal aberrations					Pericentric inversions	Polyploid cells	Percentage of cells with aberrations (X±m)
			Breaks	Fragments	Rearrangements					
					c/c	t/t	c/t			
ICR Mit. C	**24 h**	**6♂4♀ 500**	**66**	**82**	**38**	**8**	**0**	**6**	**0**	**38.42 ± 1.88**
ICR control	**24h**	**5♂1♀ 250**	**3**	**0**	**2**	**0**	**0**	**0**	**0**	**1.00 ± 0.33**
***M. spicilegus* Mit. C**	**24 h**	**6♂4♀ 500**	**49**	**50**	**5**	**2**	**0**	**2**	**0**	**21.6 ± 1.45**
***M. spicilegus* control**	**24h**	**5♂3♀ 400**	**7**	**4**	**0**	**0**	**0**	**0**	**0**	**3.25 ± 0.36**
***M. arvalis* Mit. C**	**24 h**	**10♂9♀ 902**	**64**	**32**	**0**	**0**	**0**	**4**	**0**	**10.72 ± 0.28**
***M. arvalis* control**	**24h**	**7♂ 350**	**8**	**4**	**0**	**0**	**0**	**0**	**0**	**3.43 ± 0.57**
***C. glareolus* Mit. C**	**24 h**	**2♂2♀ 165**	**9**	**6**	**1**	**1**	**0**	**1**	**0**	**10.38 ± 1.57**
***C. glareolus* control**	**24h**	**5♂5♀ 471**	**3**	**2**	**1**	**0**	**0**	**0**	**4**	**1.20 ± 0.42**
***Apodemus sp.* Mit. C**	**24 h**	**10♂8♀ 911**	**33**	**45**	**10**	**4**	**0**	**12**	**34**	**11.01 ± 0.81**
***Apodemus sp.* control**	**24h**	**5♂6♀ 498**	**9**	**4**	**0**	**0**	**0**	**0**	**0**	**2.66 ± 0.40**

Table 1. Number and frequency of chromosomal aberrations found in ICR strain laboratory mice, *M. spicilegus, M. arvalis, C. glareolus* and *Apodemus sp.* after experimental treatment with Mitomycin C (3.5mg/kg b.w./24 h).

The prevalence of breaks and fragments in the treated groups of all five species is a result of specific action of alkylating agent Mitomycin C.

Jena and Bhunya (1995) showed that Mitomycin C applied on bone marrow cells of *Gallus domesticus* provoked chromatid and isochromatid breaks, deletions and exchanges. The mechanism of action of Mitomycin C at the molecular level is due to the formation of DNA-DNA crosslinking adducts (Tomasz et al., 1987). The prevalence of breaks and fragments observed is a result of the biological action of Mitomycin C, and the number of the affected metaphases depends on the karyotype stability of the studied model species.

We found that the centromeric/centromeric fusions (c/c fusions) are more frequent in ICR mice than in all other species investigated. The rate of exchanges between non-homologous chromosomes in the mouse is particularly high. This is facilitated by the acrocentric nature of mouse's chromosomes. The acrocentric chromosomes in genus *Mus* are prone to c/c fusions because of the specific structure of the centromeric regions. This phenomenon is due to single strand's breaks in the minor SAT DNA located in the centromeric regions of mice chromosomes (Kipling et al., 1994).

In the Mitomycin C treated groups pericentric inversions – 1.33±0.36% for *Apodemus sp.* group and 1.2±0.5% for ICR mice and 3.77±0.78% polyploid metaphases (4n, 8n) in *Apodemus sp.* were also observed. In the polyploid cells some of the chromosomes were also damaged (fragments and pericentric inversions were seen).

3.2 Pb $(NO_3)_2$ clastogenicity

A total number of 49 animals from all five rodent species were treated with lead nitrate (200 mg/kg b.w.). More than 2370 metaphase plates were analized. The results are presented in Table 2.

The highest percentage of metaphases with aberrations was scored in slides of bone marrow cell population in ICR mice (11.03±0.89%). Close to these values are calculated in slides of *M. spicilegus* (9.6±0.4%). In both vole species (*M. arvalis* and *C. glareolus*) a tendency to reduce the yield of chromosomal aberrations is observed - respectively 8.93±0.61% and 8.8±0.53%. Despite the differences in absolute values of this index reported for *M. arvalis* and *C. glareolus* and laboratory mice these differences are with low level of confidence ($p \leq 0.05$).

Compound	Time after treatment	Number of metaphases scored	Type of chromosomal aberrations					Pericentric inversions	Polyploid cells	Percentage of cells with aberrations (X±m)
			Breaks	Fragments	Rearrangements					
					c/c	t/t	c/t			
ICR $Pb(NO_3)_2$	24h	6♂5♀ 550	30	11	18	2	0	0	0	11.03 ± 0.89
ICR control	24h	5♂1♀ 250	3	0	2	0	0	0	0	1.00 ± 0.33
M. spicilegus $Pb(NO_3)_2$	24h	5♂5♀ 500	22	14	11	1	0	0	1	9.60 ± 0.40
M. spicilegus control	24h	5♂3♀ 400	7	4	0	0	0	0	0	3.25 ± 0.36
M. arvalis $Pb(NO_3)_2$	24h	4♂3♀ 307	20	5	0	2	0	0	0	8.93 ± 0.61
M. arvalis control	24h	7♂ 350	8	4	0	0	0	0	0	3.43 ± 0.57
C. glareolus $Pb(NO_3)_2$	24h	7♂3♀ 500	16	16	8	3	0	0	30	8.80 ± 0.53
C. glareolus control	24h	5♂5♀ 471	3	2	1	0	0	0	4	1.20 ± 0.42
Apodemus sp. $Pb(NO_3)_2$	24h	7♂4♀ 520	20	4	0	0	0	9	12	6.00 ± 0.60
Apodemus sp. control	24h	5♂6♀ 498	9	4	0	0	0	0	0	2.66 ± 0.40

Table 2. Number and frequency of chromosomal aberrations found in ICR strain laboratory mice, *M. spicilegus, M. arvalis, C. glareolus* and *Apodemus sp.* after experimental treatment with $Pb(NO_3)_2$ (200mg/kg b.w./24 h)

The lowest percentage of metaphases with damaged chromosomes is evaluated in the group of *Apodemus sp*. (6.0±0.60%). These values are significantly lower than the percentages of cells with aberrations calculated for the other four small mammal species ($p<0.01$ to $p<0.001$).

Based on the experimental data the laboratory ICR mice and *M. spicilegus* showed a tendency of greater sensitivity to the damaging effect of $Pb(NO_3)_2$ in comparison with the other three wild species investigated. The high percentage of aberrant metaphases detected in treated *M. spicilegus* experimental group correlated with the data obtained by Topashka-Ancheva and Metcheva (1999) for *M. macedonicus* samples (8.89±2.34% for males and 10.46±0.93% for females) collected near the heavy metal polluted region of lead-zinc factory in the vicinities of Asenovgrad (Bulgaria).

The study showed that *Apodemus sp.* has significantly greater stability of the karyotype in comparison with ICR mice, *M. spicilegus, M. arvalis* and *C. glareolus* exposed to lead nitrate. This is an important conclusion in the use of *Apodemus sp.* in the ecotoxicological studies concerning the damaging influence of lead as environmental pollutant.

Chromosomal rearrangements of chromatid type - breaks and fragments predominated in all samples analyzed (from 61.1% in ICR mice up to 92% in *M. arvalis*).

The rise of chromatid type breaks and exchanges is mainly a result of double strand breaks generated in postreplicative DNA in later S phase and in G_2 phase (Hagmar et al., 2004). Chromatid type aberrations (breaks and fragments) may arise also in response to single strand breaks induced in early S phase. Chromatid breaks observed in metaphases would result from incomplete or failed repair (Pfeiffer et al., 2000). Chromatid type aberrations are usually generated by S-phase-dependent clastogens (e.g. chemical agents) (Mateuca et al., 2006). Single strand breaks resulting from lead influence were reported by Valverde et al. (2002), and Shaik et al. (2006).

The data of our experiments for the high presence of chromatid breaks in the examined small mammal species, compared with the data of other authors, provide evidence that lead is mainly S-phase-dependent clastogen. Jagetia and Aruna (1998) reported that lead induced micronuclei were observed even at very low doses. After i.p. treatment with lead nitrate, the highest increases in the frequencies of micronucleated polychromatic erythrocytes were observed after treatment with 80 mg/kg b.w. in mouse bone marrow at 12, 24 and 36 h post-treatment. These authors suggested that the increase of micronuclei might be due to the induction of DNA strand breaks by lead nitrate.

In the ICR, *M. spicilegus* and *C. glareolus* groups treated with $Pb(NO_3)_2$ metaphases with centromeric fusions were also observed (28%, 23%and 20% respectively). Centromeric/ centromeric fusions were practically not detected in *Apodemus sp.* and *M. arvalis* treated with $Pb(NO_3)_2$.

The results obtained in this study are in agreement with those of Nayak et al. (1989) who reported that lead nitrate (100-200 mg/kg b.w.) on gestational day 9 exhibited a moderate, but significantly increased sister chromatid exchanges in maternal bone marrow of ICR Swiss Webster mice. Several specific chromosomal aberrations, mostly deletions in maternal bone marrow and fetal cells were also calculated.

3.3 2,4-dichlorophenoxyacetic acid (2,4-D) clastogenicity

The quantitative data from the metaphase analysis of the bone marrow cells are summarized on Table 3.

The cytogenetic analysis carried out upon well spread metaphase plates evidenced that single administration of 2,4-D induced aberrations such as breaks, fragments and exchanges. Seldom were observed more than one aberration per analyzed metaphase. In proliferating bone marrow cell populations of all small mammal species investigated the percentages of chromosomal aberrations were approximately equal - from 4.55±0.39% in *M. spicilegus* to 5.8±0.55% in *C. glareolus*.

After triple introduction of 3.5 mg/kg b.w. 2,4-D, the damaged cells of the treated animals were at reliably higher percentages. The highest, but almost equal percentages of

chromosomal aberrations were evaluated in ICR mice (11.5±0.5%) and *Apodemus sp.* (10.33±1.08). The values were significantly higher compared to those obtained after single i.p. injection of 2,4-D ($p<0.001$). In *M. spicilegus* and *M. arvalis* experimental groups these values were lower (7.1±0.67% and 6.16±0.60%, respectively), but they were significantly higher than the data obtained after single dose only.

The types of chromosomal aberrations in samples after single and triple treatment were quite similar (breaks, fragments and centromeric/centromeric fusions, and occasionally telomeric/telomeric fusions). The telomeric/telomeric fusions are a result of terminal deletions and fusion in the region of so-called sticky edges. The higher number of metaphases with centromeric/centromeric chromosomal fusions in ICR mice and *M. spicilegus* can be explained again with the peculiarities of their acrocentric karyotype. Centromeric/centromeric fusions in the samples of *M. spicilegus* reached 60.5% after triple herbicide treatment.

Compound	Time after treatment	Number of metaphases scored	Type of chromosomal aberrations					Pericentric inversions	Polyploid cells	Percentage of cells with aberrations (X±m)
			Breaks	Fragments	Rearrangements					
					c/c	t/t	c/t			
ICR 2,4-D	**24 h**	**5♂5♀ 500**	**8**	**6**	**9**	**0**	**0**	**0**	**0**	**4,6 ± 0,43**
ICR 2,4-D triple	**24 h**	**8♂ 400**	**14**	**16**	**16**	**0**	**0**	**0**	**0**	**11,5 ± 0,5**
ICR control	**24h**	**5♂1♀ 250**	**3**	**0**	**2**	**0**	**0**	**0**	**0**	**1,0 ± 0,33**
***M. spicilegus* 2,4-D**	**24 h**	**4♂7♀ 550**	**12**	**6**	**6**	**1**	**1**	**0**	**1**	**4,55 ± 0,39**
***M. spicilegus* 2,4-D triple**	**24 h**	**5♂5♀ 550**	**10**	**5**	**23**	**1**	**0**	**0**	**0**	**7,1 ± 0,67**
***M. spicilegus* 2,4-D control**	**24 h**	**8♂2♀ 500**	**3**	**10**	**5**	**0**	**0**	**0**	**1**	**3,67 ± 0,61**
***M. arvalis* 2,4-D**	**24 h**	**5♂5♀ 491**	**19**	**2**	**2**	**0**	**0**	**0**	**2**	**4,72 ± 0,48**
***M. arvalis* 2,4-D triple**	**24 h**	**3♂6♀ 430**	**13**	**10**	**0**	**0**	**0**	**0**	**1**	**6,16 ± 0,60**
***M. arvalis* control**	**24h**	**7♂ 350**	**8**	**4**	**0**	**0**	**0**	**0**	**0**	**3.43 ± 0.57**
***C. glareolus* 2,4-D**	**24 h**	**4♂6♀ 500**	**19**	**7**	**2**	**1**	**0**	**0**	**2**	**5,8 ± 0,55**
***C. glareolus* control**	**24h**	**5♂5♀ 471**	**3**	**2**	**1**	**0**	**0**	**0**	**4**	**1,2 ± 0,42**
***Apodemus sp.* 2,4-D**	**24 h**	**6♂6♀ 612**	**15**	**11**	**4**	**1**	**0**	**0**	**4**	**5,0 ± 0,52**
***Apodemus sp.* 2,4-D triple**	**24 h**	**4♂2♀ 300**	**7**	**24**	**0**	**0**	**0**	**0**	**13**	**10,33 ± ,08**
***Apodemus sp.* 2,4-D control**	**24 h**	**6♀ 300**	**4**	**4**	**1**	**0**	**0**	**0**	**1**	**3,0 ± 0,45**

Table 3. Number and frequency of chromosomal aberrations found in ICR strain laboratory mice, *M. spicilegus, M. arvalis, C. glareolus* and *Apodemus sp.* after experimental treatment with 2,4-dichlorophenoxyacetic acid 2,4-D (3,5 mg/kg b.w.)

Calculated as percentage of the total number of damaged cells, the amount of the chromatid type aberrations (breaks and fragments) ranged from 60% in ICR mice up to 100% in *M. arvalis* and *Apodemus sp.* after triple treatment with 2,4-D.

We found that 2,4-dichlorophenoxyacetic acid exhibits moderate damaging effect on the chromosomes of the studied small mammal species. This damaging effect is increased because of the triple herbicide introduction. These results are in agreement with the data of Amer and Aly (2001), who found a significant increase in the percentage of chromosomal aberrations in bone marrow and spermatocyte cells after oral administration of 3.3 mg/kg b.w. 2,4-D for three and five consecutively introduced applications of the herbicide. At the same time, a single dose of 1.7 mg/kg and 3.3 mg/kg

b.w. did not show a significant increase of the chromosomal aberrations in bone marrow and spermatocyte cells of the male Swiss mice. These authors found that 2,4-D induce structural and numerical chromosomal aberrations in bone marrow cells and a dose-dependent relationship in the percentage of metaphases with chromosomal aberrations (6.8±0.73% 24 hours after single oral treatment with 3.3 mg/kg b.w.). Our results were quite similar – 4.6±0.43% after single i.p injection in ICR mice and 11.5±0.5% after triple introduction of the herbicide.

In earlier studies Venkov et al. (2000) in C57Bl mice treated i.p. with 3.5 mg/kg 2,4-D found that this herbicide exhibits a positive reply for chromosomal aberrations and significantly reduced mitotic activity with *in vivo* test. These authors found that 2,4-D induced 5.50±1.90% chromosomal aberrations 24 hours after i.p. treatment with significant prevalence of centromeric fusions, but also observed that 2,4-D had weaker clastogenic effect compared to the positive control Mitomycin C (23.35±0.95%). From the results of their experiment, it can be suggested that the genetic damage caused by phenoxy herbicide should not be ignored. We confirmed and enlarged their results in our experimental design that in all five small mammal species investigated the equal dose of 3.5mg/kg 2,4-D possessed significantly lower clastogenic effect compared to the same dose of the alkylating agent Mitomycin C. Our results obtained in *in vivo* investigations support the results of Gandhi et al. (2003), that a potential genetic hazard exists because of the widespread use of 2,4-D in agroecosystems. Since the genetic damage may result from the exposure to agricultural chemicals in the environment, there is a further need to evaluate the potential hazard that 2,4-D may exhibit to animal species living in the wild.

3.4 THF clastogenicity

The data of the cytogenetic analysis conducted on the treated bone marrow cells with 3.8 mg/kg THF on small mammal species is presented on Table 4.

Compound	Time after treatment	Number of metaphases scored	Type of chromosomal aberrations					Pericentric inversions	Polyploid cells	Percentage of cells with aberrations (X±m)
			Breaks	Fragments	Rearrangements					
					c/c	t/t	c/t			
ICR THF	**24h**	**4♂4♀ 400**	**19**	**15**	**23**	**2**	**0**	**0**	**0**	**14.75±0.92**
ICR control	**24h**	**5♂1♀ 250**	**3**	**0**	**2**	**0**	**0**	**0**	**0**	**1.00 ± 0.33**
***M. spicilegus* THF**	**24h**	**4♂8♀ 600**	**20**	**19**	**6**	**0**	**0**	**0**	**0**	**7.50 ± 0.44**
***M. spicilegus* control**	**24h**	**5♂3♀ 400**	**7**	**4**	**0**	**0**	**0**	**0**	**0**	**3.25 ± 0.36**
***M. arvalis* THF**	**24h**	**4♂5♀ 384**	**20**	**5**	**0**	**1**	**0**	**0**	**2**	**6.93 ± 0.52**
***M. arvalis* control**	**24h**	**7♂ 350**	**8**	**4**	**0**	**0**	**0**	**0**	**0**	**3.43 ± 0.57**
***C. glareolus* THF**	**24h**	**4♂6♀ 500**	**19**	**12**	**3**	**3**	**0**	**0**	**2**	**7.40 ± 0.43**
***C.glareolus* control**	**24h**	**5♂5♀ 471**	**3**	**2**	**1**	**0**	**0**	**0**	**4**	**1.20 ± 0.42**
***Apodemus sp.* THF**	**24h**	**11♂3♀ 621**	**19**	**23**	**2**	**0**	**0**	**0**	**7**	**6.85 ± 0.41**
***Apodemus sp.* control**	**24h**	**5♂6♀ 498**	**9**	**4**	**0**	**0**	**0**	**0**	**0**	**2.66 ± 0.40**

Table 4. Number and frequency of chromosomal aberrations found in ICR strain laboratory mice, *M. spicilegus, M. arvalis, C. glareolus* and *Apodemus sp.* after experimental treatment with THF (3.8mg/kg b.w./24 h).

The results showed that the highest percentage of cells with aberrant chromosomes was evaluated in ICR mice (14.75±0.92%). Bone marrow cells of four other species studied possessed great similarity in their reaction to the genotoxin applied. The data about the absolute values for the percentage of cells with aberrations in *M. spicilegus*, *M. arvalis*, *C. glareolus* and *Apodemus sp*. did not significantly differ ($p>0.05$). In all samples analyzed chromatid-type aberrations (breaks and fragments) were predominating. The highest percentages of breaks and fragments among all aberrations scored were observed in *M. arvalis* (96%) and *Apodemus sp.* (95%), and the lowest in the ICR mice group (57.6%). In the ICR group high percentage of cells with chromosomal exchanges were scored (42.4% aberrant cells with c/c and t/t fusions).

It should be noted however that values for chromosomal aberration obtained after treatment with THF were significantly lower than those with Mitomycin C. These findings suggest a moderate clastogenic effect of the studied genotoxin. There was no statistical significance in the values of aberrant metaphases obtained in experimental groups treated with THF and $Pb(NO_3)_2$ ($p>0.05$). ICR mice, *M. spicilegus* and *M. arvalis* showed higher chromosome sensitivity to the THF effect compared to the herbicide 2,4-D ($p<0.001$; $p<0.01$, respectively). Such dependency was not observed in the aberrant metaphases in *C. glareolus* and *Apodemus sp*. after single i.p. treatement with 2,4-D.

The results obtained in our cytogenetic study evidence that THF have a clastogenic effect and confirm previous data (Gamer et al., 2002) showing an increase of sister chromatid exchanges and chromosomal aberrations following an i.p. treatment with that genotoxin.

4. Conclusion

The effect of various genotoxins on the chromosome set has been intensively studied. Studies on the structural changes in the karyotypes of small mammal species are of a great interest. In this context we investigated the effect of certain genotoxins (Mitomycin C, Lead nitrate, herbicide 2,4-D and THF) on the chromosome integrity of ICR laboratory mice, *M. spicilegus*, *M. arvalis*, *C. glareolus* and *Apodemus sp.* in experimental conditions. Original data about the amount and the type of chromosome aberrations were obtained.

All four wild small mammal species (*M. spicilegus*, *M. arvalis*, *C. glareolus* and *Apodemus sp.*) and the ICR laboratory mice used in this experimental design have responded by changes in chromosomal structure after the intraperitoneal injection of all genotoxins applied.

All the injected compounds caused predominantly chromatid breaks and fragments in small mammal species investigated. Chromosomal exchanges (c/c and t/t fusions) were characteristic mainly for ICR mice and *M. spicilegus* because of their acrocentric type of chromosomes.

The highest karyotype sensitivity to the genotoxins applied was detected in the ICR mouse karyotype, followed by its closely related species *M. spicilegus*.

Both vole species *M. arvalis* and *C. glareolus* showed a similar chromosomal fragility towards the action of most genotoxins applied (Mitomycin C, $Pb(NO_3)_2$ and THF). The karyotype of *Apodemus sp.* showed relatively highest chromosomal stability to the genotoxic action of lead nitrate and Mitomycin C. The percentages of metaphases with aberrant chromosomes in

Apodemus sp. groups after treatment with the herbicide 2,4-D and THF were similar to those of *M. spicilegus*, *M. arvalis* and *C. glareolus*.

The highly pronounced karyotype reaction of *M. spicilegus* to the genotoxins applied and its attachment to agroecosystems would allow the use of this species in genetical monitoring. *M. spicilegus* also has an advantage because of its relatively small chromosomal set (2n=40) and acrocentric type of chromosomes, which allows fast and accurate evaluation of the arising structural chromosomal aberrations. As we found that the chromosome fragility of both *Mus* species (laboratory ICR mice and *M. spicilegus*) was rather similar, the laboratory mouse may also be used successfully in a relatively accurate assessment concerning the genotoxic effects of various xenobiotics introduced in one way or another in agroecosystems.

In so far as the three species – *M. arvalis*, *C. glareolus* and *Apodemus sp.* also react with changes in the structure of their chromosomes under influence of various genotoxins, each of them can be used for the needs of the impact as well as the background genetic monitoring.

Finally, chromosome set of the studied model species possess different sensitivity to the effect of the genotoxin applied.

In all small mammal species investigated the type of chromosomal aberrations observed depends on the chemical nature of the mutagen applied, while the amount of the damaged cells is determined by the karyotype stability.

The specific sensitivity of the chromosomes observed in all used wild small mammal species imposed as zoomonitors is a very important characteristic that must be borne in mind when these species are used for environmental genetic risk assessment.

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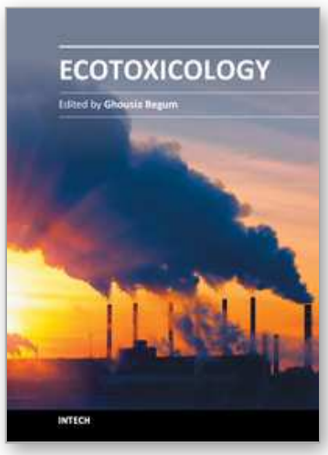

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This is a good book on upcoming areas of Ecotoxicology. The first chapter describes genotoxicity of heavy metals in plants. The second chapter offer views on chromatographic methodologies for the estimation of mycotoxin. Chapter three is on effects of xenobiotics on benthic assemblages in different habitats of Australia. Laboratory findings of genotoxins on small mammals are presented in chapter four. The fifth chapter describes bioindicators of soil quality and assessment of pesticides used in chemical seed treatments. European regulation REACH in marine ecotoxicology is described in chapter six. X-ray spectroscopic analysis for trace metal in invertebrates is presented in chapter seven. The last chapter is on alternative animal model for toxicity testing. In conclusion, this book is an excellent and well organized collection of up dated information on Ecotoxicology. The data presented in it might be a good starting point to develop research in the field of ECOTOXICOLOGY.

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