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The Effect of Environmental Factors on Sister Chromatid Exchange Incidence in Domestic Horse (*Equus caballus*) Chromosomes

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The SCE test is often used as a sensitive and reliable technique in the biomonitoring of genotoxicity of mutagenic and carcinogenic agents. This study analysed the frequency of sister chromatid exchange in domestic horse chromosomes depending on the habitat and age of the analysed horses. The chromosome preparations were obtained from an *in vitro* culture of peripheral blood lymphocytes stained using the FPG technique. Both the habitat and the age significantly influence SCE frequency. A higher SCE incidence was observed in horses that lived in a large urban agglomeration than in those from the country. Also, a higher SCE incidence was identified in the group of horses above 6 years of age in comparison with the younger ones. Additionally, the frequency of SCEs in the first, second and third chromosomes and the X sex chromosome were analysed in detail. More exposed to the effect of environmental pollutants, the horses from the urban environment developed more double and triple SCEs in comparison with the village horses. The urban horses also developed quadruple SCEs, in addition to the less frequent exchanges.

Key words: Horse, habitat, environmental factor, chromosome instability, sister chromatid exchange.

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Chromosomes are sensitive structures vulnerable to damage from detrimental environmental factors. The use of cytogenetic techniques in environmental biomonitoring helps obtain information on the level of exposure to genotoxic factors and evaluate the effect of environmental pollution to provide an assessment of the actual threat to man and animals. The basic test used in chromosome instability assessments is the sister chromatid exchange (SCE) test. It enables the detection of DNA damage caused by mutagenic and genotoxic factors. Sister chromatid exchanges can be observed in metaphase chromosomes as discontinuities in the pigmentation of the pattern of two chromatids that belong to the same chromosome. SCEs arise during or immediately after DNA replication as a result of matrix damage, when DNA alterations have not been repaired before the cell enters the S phase of the cell cycle (WÓJCIK *et al.* 2004). DNA can suffer damage in one of its strands or in both strands. Dysfunctional DNA damage repair mechanisms contribute to SCE incidence. SCEs occur when the intermediate product of the Holliday connection is distributed in one of two directions. A change in direction causes inconsistencies and errors that can be repaired with various corrective systems, e.g. non-homologous end-joining (WILSON & THOMPSON 2007). This process is inaccurate and often causes modifications of the DNA sequence that may be observed as sister chromatid exchanges. SONODA *et al.* (1999) and WILSON & THOMPSON (2007) think that homologous recombination is one of the chief mechanisms responsible for sister chromatid exchanges in vertebrate cells. Unfortunately, the molecular mechanism of SCE generation and its biological significance still remain to be determined, although many researchers have undertaken relevant analyses.

Among many farm animal species that are of interest to scientists, the ones most frequently used in cytogenetic investigations include cattle, sheep, goats and pigs. Due to the use of horses mainly for recreation, sports, and as draught animals, the horse karyotype has not been so well defined as in other animals. The diploid number of horse chromosomes is 64. In the morphologically differenti-

ated karyotype, 27 chromosomes are two-armed and 37 are acrocentric. The banding pattern standard includes definitions of the CBG, GTG, RBG and NOR-related bands (Richer *et al.* 1990; Bowling *et al*. 1997). Studies of the horse genome and karyotype have been undertaken to analyse their evolutionary aspect and compare them with the genomes of other mammal species (Caetano *et al.* 1999; Wade *et al.* 2009). Few publications address chromosome instability in horses (Rubes *et al.* 1992; Wójcik *et al.* 2011) and cytogenetic analyses undertaken by scientists mainly concern irregularities in chromosome number or structure (Bugno & Słota 2007; Lear & Bailey 2008; di Meo *et al.* 2009).

The present study was aimed at evaluating the influence of the environment on sister chromatid exchange incidence in the domestic horse (*Equus caballus*) karyotype.

Material and Methods

The study was carried out according to the guidelines of the III Ethical Committee in Warszawa (No 37/2011 from the 22 June 2011).

The analyses were carried out for a group of 40 differently aged Polish cold-blooded horses from two different environments. The horses came from eastern Poland. The first group (Group 1) was represented by horses that were kept in a village 15km away from the city (20 horses), the second group (Group 2) was constituted by horses that lived in a large industrial city (20 horses). Both groups of horses stayed in these conditions from birth. Considering age differences among the horses in Group 1 and 2, they were divided into 2 age groups: up to 6 years and above 6 years of age. The chromosome preparations were obtained from an *in vitro* culture of peripheral blood lymphocytes to which BrdU (10μg/ml) was added in the 24th hour of culture. The chromosomes were stained using the FPG technique (Kihlman & Kronborg 1975). The staining procedure involved the following stages: one-hour 0.01% RNase treatment at 37°C, one-hour incubation in a 0.5×SSC solution (0.75M sodium chloride + 0.075M sodium citrate; pH=7.0) including Hoechst's solution (the basic solution: 0.5 mg Hoechst 33258/1ml ethanol), one-hour UV exposure, 24-hour incubation at 4°C in darkness, half-hour UV treatment, two-hour incubation at 58°C and one-hour Giemsa staining.

The preparations were analysed under an Olympus BX 50 light microscope. A detailed imaging analysis was carried out using the Multiscan image analysis system, the Karyotype software, and graphic software compatible with the system. 30 metaphases were analysed for each animal. Additionally, the first, second and third chromosomes and the X chromosome were analysed in detail. The number and sites of SCEs in the particular chromosomes were identified. Moreover, the numbers of single and multiple SCEs were determined. The results where processed statistically. The effects of the environment and the age on SCE frequency were studied using bivariate analysis of variance. The correlation between chromosome length and the number of SCEs identified in the chromosomes was determined using the Pearson correlation coefficient.

Results

In the analysed horse population, the mean SCE/cell in the 1200 investigated cells was 5.15±1.44. Higher SCE frequency was observed in the group of horses living in the city as compared with those from the country (Figs 1, 2). The mean number of SCEs/cell in the group of horses kept in the country was 4.18±1.07. On the other hand, the mean number of SCEs/cell in the group of horses from urban agglomerations was 6.09±1.09. A higher mean number of SCEs/cell was observed in the group of horses of more than 6 years of age

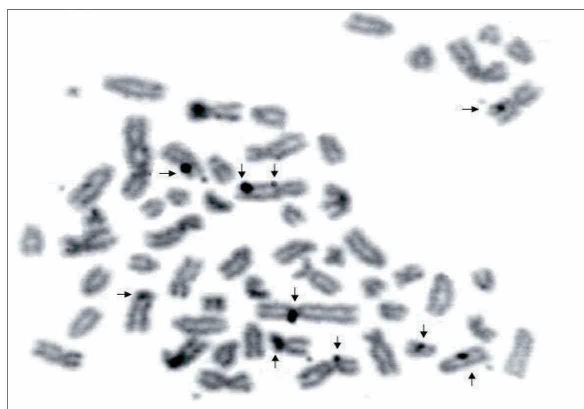

Fig. 1. Chromosome metaphase plates of the rural horses. Arrows indicate SCEs.

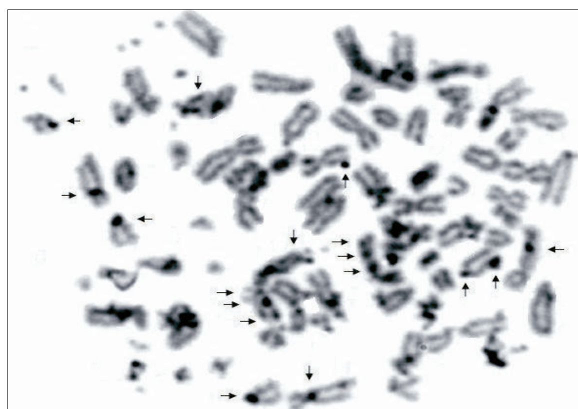

Fig. 2. Chromosome metaphase plates of the urban horses. Arrows indicate SCEs.

Table 1

SCE distribution in the selected horse cells and chromosomes, specifically chromosomes 1, 2, 3 and X

Chromosome	Number of cells	Number of SCE/chromosome	
		Total	Mean ± SD
1	1200	3108	2.59±0.99
2	1200	2047	1.71±0.70
3	1200	2000	1.68±1.01
X	1200	2245	1.88±0.67

(5.77±1.35) as compared with the first age group (4.62±1.31). After bivariate analysis of variance, both environment and age were found to have significant effects on SCE incidence (P<0.01).

The frequencies of SCEs in the first, second and third chromosomes as well as the X sex chromosome, analysed in detail, are presented in Table 1. The highest number of SCEs was observed in the first and the lowest in the third chromosome. The number of SCEs identified was proportional to chromosome length – the longer the chromosome, the more exchanges that occurred. A positive correlation between the characteristics was determined. The Pearson correlation coefficient was 0.89 (P<0.01). More SCEs were also identified in the long *q* chromosome arm (56%) than in the short *p* arm (44%) of the analysed chromosomes. Sister chromatid exchanges were observed in the proximal, interstitial and distal areas of the chromosomes. We observed the most exchanges in the interstitial part of the analysed chromosomes (47%), followed by the distal (33%) and proximal (20%) regions. The SCE distribution was similar in the detailed analysis of chromosomes 1, 2, 3 and X. The highest number of exchanges was observed in the interstitial region, followed by the distal and proximal ones (Fig. 3).

The study identified single, as well as double, triple and quadruple sister chromatid exchanges in the chromosomes. The percentage share of the exchanges in the particular horse groups varied. The chromosomes of the rural horses were found to have the highest number of single exchanges, followed in a descending order by double and triple SCEs. The chromosomes of the horses in the urban areas, in turn, were observed to undergo predominantly double exchanges, followed by single, triple and few quadruple SCEs (Fig. 4).

Discussion

The SCE test is often used as a sensitive and reliable technique in assessments of the effect of dysfunction-causing xenobiotics and pollutants on man and animals. They are exogenous substances that enter the organism along with consumed food and beverages or inhaled air.

Most bioindication studies have concerned man. However, studies examining animals are also

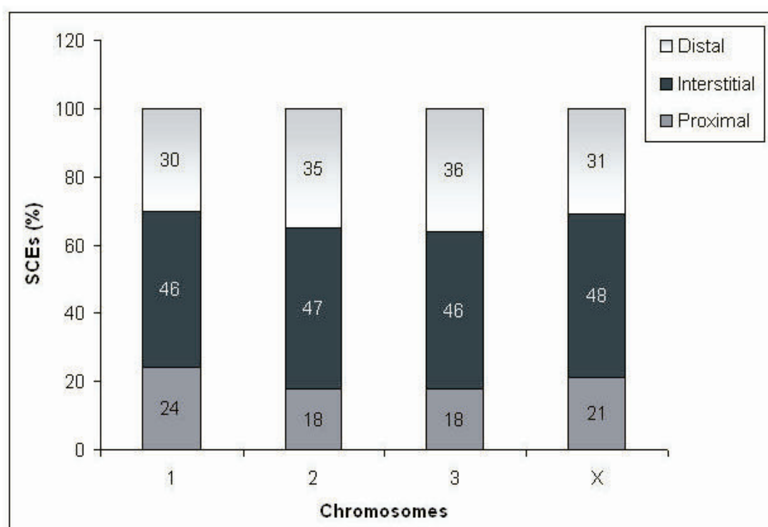

Fig. 3. Percentage distribution of SCEs in the analysed chromosomes.

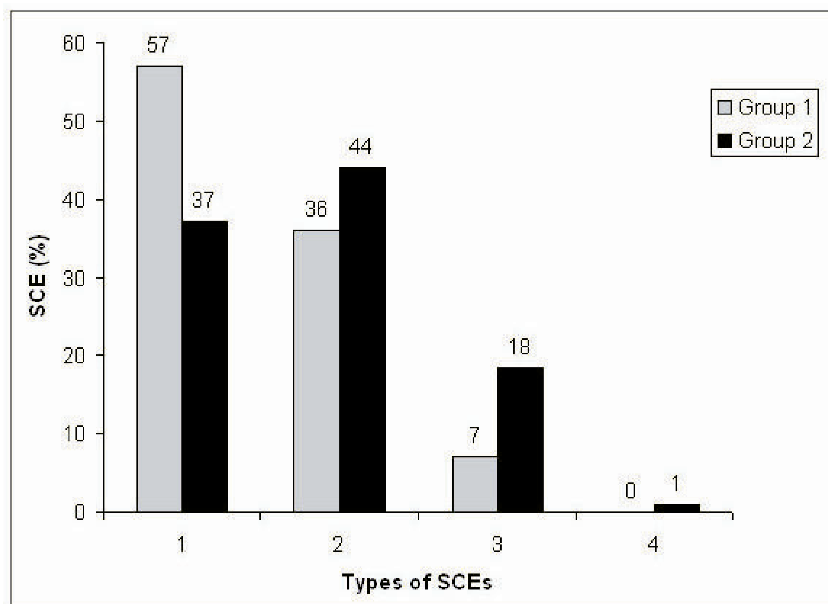

Fig. 4. The percentage share of the particular types of SCE in the rural (group 1) and urban (group 2) horses.

available (PARADA & JASZCZAK 1993; RUBES *et al.* 1992; PERUCATTI *et al.* 2006). Such analyses use different tests to determine the toxicity of various environmental factors on the basis of reactions of living organisms. RUBES *et al.* (1992) studied the effect of environmental pollutant induction in various animal species including cows, horses, pigs and deer in highly industrialised areas close to numerous factories. They observed rising SCE frequency in herbivorous animals which can be good biomarkers of the exposition, effect and susceptibility to environmental pollutants. PERUCATTI *et al.* (2006) analysed the toxic effect of dioxins on sheep, cattle and the river buffalo. Dioxins belong to the most toxic and carcinogenic substances generated during chemical reactions in industry or spontaneous ones triggered by the burning of various organic compounds. PERUCATTI *et al.* (2006) observed an increase in chromosome damage, higher SCE frequency and higher mortality in animals that lived in the vicinity of large cities as compared to animals living at a substantial distance from urban agglomerations. Differences in SCE frequency may stem from intensive or extensive animal breeding (CATALAN *et al.* 1995; PERETTI *et al.* 2006). In both cases animals are maintained on different feeding regimes. CATALAN *et al.* (1995) observed higher SCE frequency in animals grazing on pastures than in those kept in closed rooms (stabled). According to these authors, higher SCE frequency in pastured animals may be the consequence of greater exposure to environmental pollution. Differences in SCE incidence were also observed in our study between the horse groups. The urban horses were found to have a higher SCE frequency than the group of horses from the rural areas. The rural horses were not exposed to considerable environmental pollution, e.g. dust, gases, fumes produced by various chemical reactions, burning of wood or organic combustion at various industrial plants, factories and a large number of individual households. The study shows that horses are good environmental biomarkers. The applied cytogenetic method is particularly useful, as it enables detection of DNA damage caused by various noxious genotoxic and carcinogenic substances.

SCE incidence is affected by the age of animals. Older animals tend to have increasingly more SCEs (SINHA *et al.* 1985; LAZUTKA *et al.* 1994; PERETTI *et al.* 2006; HUSUM *et al.* 2008). Our study confirmed this regularity. A higher number of sister chromatid exchanges was identified in the group of horses above 6 years of age.

In order to verify the negative influence of mutagenic and genotoxic pollutants on living organisms and visualise the concomitant DNA damage, *in vitro* cell cultures are supplied with nitric base analogues, e.g. the thymine analogue – BrdU. Unfortunately, BrdU is a strong SCE inducer that is incorporated in place of thymidine. Spontaneous SCE incidence depends on the BrdU dose. Differing opinions exist as to the BrdU dose that should be applied. WILSON & THOMPSON (2007) claim that the BrdU level should be very low or close to zero. The optimal BrdU dose is 5-30 μg/ml (LEINBENGUTH & THIEL 1986; DI BERARDINO *et al.* 1996; PERETTI *et al.* 2006). In order to induce SCE, a 10 μg/ml BrdU dose was used in the present study.

Chromosome length was a factor that influenced SCE frequency. The frequency of the exchanges

was proportional to chromosome length – the longer the chromosome, the more numerous the exchanges (LATT 1974; IANNUZZI *et al.* 1991; ARIAS 2000; CORRANO & WOLFF 1975; VIJH *et al.* 1991). This applies for all analysed species: humans, goats, cattle and chickens. On the other hand, DI MEO *et al.* (1993; 2000) observed that SCE distribution in the chromosomes of different species was irregular. The authors (1993; 2000) identified higher or lower SCE frequency in certain chromosomes in comparison with chromosome length. Our study revealed that the number of SCEs was positively correlated with the relative lengths of the analysed chromosomes.

The location of SCE within a chromosome is probably not accidental. In the Chinese hamster (MARTIN & PRESCOTT 1964), the rat (GIBSON & PRESCOTT 1972) and the mouse (LIN & ALFI 1976), SCEs were predominantly located in the centromeric (proximal) regions. In turn, LATT (1974) observed the fewest SCEs in the centromeric region in human chromosomes. LATT (1974) and IANNUZZI *et al.* (1991) identified the most SCEs in the G bands of chromosomes. CORRANO & WOLFF (1975) detected far more exchanges in the heterochromatin region than in the euchromatin area. Furthermore, the scientists observed higher SCE incidence in the euchromatin and heterochromatin binding sites. The reason for different SCE locations in particular individuals may be species-specific differences of heterochromatin configuration in the centromeres (LINDAHL 1993). Our study revealed the highest number of SCEs in the interstitial parts of the chromosomes. These were the areas in which heterochromatin was bordered by euchromatin. Heterochromatin in horses is located in the proximal area (RICHER *et al.* 1990; BOWLING *et al.* 1997; WÓJCIK *et al.* 2009). The size of constitutive heterochromatin chunks in the proximal regions of the horse chromosomes ranged from 13% to 44% (WÓJCIK *et al.* 2009). It was in these chromosome regions that the fewest SCEs were observed. SCE frequency in the terminal region was 33%. The terminal part of a chromosome which includes the telomeres and subtelomeres is 5-10 times more vulnerable to damage caused by oxidative stress than the other regions (KAWANISHI & OIKAWA 2004). As a result of exposure to oxidative stress, the number of discontinuities in one-strand telomeric DNA rises. When slight damage occurs, the cell stops dividing and launches DNA repair mechanisms. Any shortcomings in DNA repair are identified as SCE. RUDD *et al.* (2007) detected high SCE frequency in the terminal chromosome region, amounting to 17%.

There are few studies of multiple sister chromatid exchanges in chromosomes. The phenomenon of elevated exchange frequency with multiple SCEs was observed in humans in various syndromes, e.g. the Bloom syndrome (YOUSSOUFIAN & PYERITZ 2002; AMOR-GUERET 2006). PERETTI *et al.* (2008) analysed SCEs in Mediterranean Italian buffaloes affected by limb malformation (transversal hemimelia). They observed double and triple exchanges. They found that the double exchanges were more frequent than the triple ones. The present study also identified double and triple SCEs in urban horses. Apart from these, rare quadruple exchanges were also observed. On the other hand, quadruple SCEs were not detected in rural horses.

In conclusion, the SCE test employed in this study is a very sensitive method for detecting early genotoxic chromosome defects occurring in the replication process that are caused by dysfunctional repair mechanisms. Our results indicate the usefulness of the SCE test for the evaluation of the effect of genotoxic factors in animals.

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