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Spontaneous DNA Damage in Lymphocytes of Fox Species

Anna GRZESIAKOWSKA, Olga SZELESZCZUK, Marta KUCHTA-GŁADYSZ, and Agnieszka OTWINOWSKA-MINDUR

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Original article

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The comet assay is a common tool used to evaluate cellular genetic instability at the DNA level. It determines the effect of physical, chemical and environmental factors on DNA, and thus shows the body's individual resistance to harmful substances. The karyotypes of blue and silver foxes and their inter species hybrids are characterized by morphological and structural variation. This variation is partly attributable to the presence of chromosomal polymorphism, which may significantly influence the stability of genetic material in the cells of these species. The aim of this study was to evaluate genetic material stability in selected Canidae species. To this end, analyses using the alkaline comet assay were performed. The interaction between sex and species was significant ($p<0.05$) for percentage of head DNA, tail DNA and Olive Tail Moment, and highly significant ($p<0.01$) for tail length and Tail Moment. The chromosomal polymorphism observed in the somatic cells of original species (blue and silver fox), does not affect the stability of the genetic material in the interspecific fox hybrids.

Key words: Foxes, comet assay, DNA integrity.

Anna GRZESIAKOWSKA✉, Olga SZELESZCZUK, Marta KUCHTA-GŁADYSZ. Department of Animal Anatomy, Institute of Veterinary Medicine, University of Agriculture in Krakow, Mickiewicz Av. 24/28, 30-059 Kraków, Poland.
E-mail: grzesiakowska.anna.ur@gmail.com
Agnieszka OTWINOWSKA-MINDUR, Department of Genetics and Animal Breeding, University of Agriculture in Krakow, Mickiewicz Av. 24/28, 30-059 Kraków, Poland.

The 1970s and 1980s witnessed an increasing number of studies on species of the Canidae family. Ever since the presence of chromosomal polymorphism was discovered in species such as the arctic fox (MÄKINEN 1985a) (*Vulpes lagopus* synonym *Alopex lagopus*) (LOHI *et al.* 2015), the silver fox (*Vulpes vulpes*) (MÄKINEN 1985b) and the Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*) (ŚWITOŃSKI *et al.* 2003), these animals have been the subject of extensive genetic and biological research. Genome mapping of domestic animals became popular in the early 1990s. As a result, the karyotype of the common ancestor of the order Carnivora was reconstructed as 2n=42 chromosomes (ŚWITOŃSKI *et al.* 2003). Chromosomal polymorphism in foxes is associated with a variable chromosome number in the karyotype. In the blue fox genome, the polymorphism is related to a variable diploid number of chromosomes (2n=48, 49 or 50) and is caused by the occurrence of centric fusions. The karyotype polymorphism of the silver fox is associated with the presence of additional B chromosomes (GRZESIAKOWSKA *et al.* 2017). In interspecific fox hybrids both types of chromosomal polymorphism of the original species can be observed. The fusion of two unstable karyotypes from both parents could generate cellular mosaicism (SZELESZCZUK *et al.* 2018).

It is common practice in breeding to create interspecific hybrids of animals. The resulting individuals may show increased body weight, better resistance to disease, improved yield and productivity (SHORT 1997). Interspecific hybrids of the blue and silver foxes are most often created by crossing female arctic (blue) foxes with male silver foxes. The resultant offspring are viable but infertile, because meiotic chromosome disturbances lead to the formation of abnormal gametes (MÄKINEN &

GUSTAVSSON 1982; BUGNO-PONIEWIERSKA *et al.* 2015). Interspecific fox hybrids are produced on account of the high quality of their fur coat, which is short but fluffy. It resembles blue fox fur in structure and silver fox fur in color. At the same time, the hybrid has a relatively large body size as in the silver fox (BUGNO-PONIEWIERSKA *et al.* 2015).

Recent years have seen increased interest in research concerning the impact of various environmental, genetic or lifestyle factors on DNA integrity in humans (FENECH *et al.* 1999), but also in plants and animals. A variety of factors could cause genomic instability, which might be observed as chromosomal instabilities or fragmentation of DNA strands in the cell nucleus by comet assay (SCGE, single cell gel electrophoresis). Chromosomal instabilities (CIN) are evaluated using the micronucleus test, the sister chromatid exchange test, the bleomycin test, and the comet assay. The main chromosomal instabilities subjected to most analyses are isochromosomes, dicentric chromosomes, micronuclei, sister chromatid exchanges, and fragile sites (ŚWITOŃSKI *et al.* 2006; GEIGL *et al.* 2008; KOZŁOWSKA & ŁACZMAŃSKA 2010; CZUBASZEK *et al.* 2014). The analysis of chromosomal instability is of utmost importance for cytogenetic diagnosis as it evaluates DNA integrity in chromosomes and chromatin structure.

Our study was aimed to determine if and how the presence of chromosomal polymorphism in blue and silver foxes influences stability of genetic material in somatic cells of the interspecific hybrids of these species.

Material and Methods

The study was conducted on 36 farmed foxes belonging to 3 breeds: blue foxes (B) (*Vulpes lagopus* synonym *Alopex lagopus*), silver foxes (S) (*Vulpes vulpes*) and their interspecific hybrids (H) (*Vulpes lagopus* x *Vulpes vulpes*). An experimental group of 12 animals (6 males and 6 females) was established for each breed. All the animals were young in age, between 1-2 years old.

The study used lymphocytes obtained from *in vitro* culture of peripheral whole blood, obtained during veterinary examinations from *V. cephalica antebrachi*. To perform the SCGE test, lymphocytes were isolated using Histopaque 1077, and cells were suspended on a slide between two layers: 0.5% LMPA (Sigma Aldrich) and 0.5% NMPA (Sigma Aldrich). Next, 24 h cell lysis was performed in alkaline buffer (2.5 M NaCl, 100 mM Na_2EDTA, 0.4 M Tris-HCl, 1% sodium N-lauroyl sarcosine, 10% Triton X-100, 1% DMSO, pH = 10). Electrophoretic separation was performed in TBE buffer (10N NaOH, 200 mM EDTA, pH≥13) at 25 V, 300 mA for 20 min, and neutralization using 0.4 M Tris-HCl (Sigma Aldrich). Detection was performed with 200 µg/ml ethidium bromide (SINGH *et al.* 1988).

DNA integrity in each animal was evaluated based on measurement of 50 cells. Sensitivity of DNA to endogenous damage was assessed based on the percentage of head DNA (HD), tail DNA (TD), tail length (TL), and the values of Tail Moment (TM) and Olive Tail Moment (OTM). These parameters measure DNA migration from the nucleus.

Microscopic analysis and photographic documentation were made under a Zeiss Imager A2 epifluorescence microscope fitted with a Zeiss AxioCam MRc5 camera. The test was evaluated with CASP 1.2.3b software. In each preparation, 50 cells that met the criterion of comet assay evaluation were assessed (SINGH *et al.* 1988).

All analyses were performed using SAS software (SAS, 2014). Data were evaluated for normality before analysis using the Shapiro-Wilk test. The nonconforming data were Ln transformed before further analysis, but actual mean values are presented in tables. The following linear model was fitted using the MIXED procedure in SAS (2014):

$$Y_{ijk} = \mu + SEX_i + SP_j + (SEX \times SP)_{ij} + \varepsilon_{ijk}$$

where:

Y_{ijk} – k-th observation (% head DNA; % tail DNA; tail length; TM; OTM) from i-th sex and j-th species,

μ – overall mean,

SEX_i – effect of i-th sex (i = 1, 2),

SP_j – effect of j-th species (j = 1, 2, 3),

$(SEX \times SP)_{ij}$ – effect of interaction between sex and species,

ε_{ijk} – residual effect.

Results from 50 cells for each animal were included in the analysis. The significance of differences between means was determined by the Tukey-Kramer test. All values were expressed as mean ± standard deviation (SD).

Results

A total of 600 cells were analysed for the group of tested animals in each breed. Figure 1 presents cells with different degrees of DNA disintegrity. Cell DNA integrity was evaluated based on the five parameters of the comet assay: the percentage of head (HD) and tail DNA (TD), tail length (TL), and the values of Tail Moment (TM) and Olive Tail Moment (OTM).

The interaction between sex and species was significant ($p < 0.05$) for percentage of head DNA, tail

DNA and Olive Tail Moment. Highly significant differences were observed between sexes for all the analysed parameters ($p<0.01$). The males were more sensitive to DNA damage for all parameters. The mean percentage of TD was 22.46±21.32% in males and 20.09±18.06% in females (Table 1). Means within sex appeared similar for tail length, which was 126.63±104.11 μm in males and 115.08±86.91 μm in females. In addition, males showed higher mean values for TM and OTM. Detailed results are shown in Table 1.

The same parameters (HD, TD, TL, TM and OTM) were considered for species comparisons (Table 2). The best preserved DNA integrity was observed for material obtained from group H (hybrids), for which the percentage of tail DNA was 8.31±5.98%. Thus, the SCGE assay parameters for the hybrids were 91.69±5.98% for HD and 34.81±24.04 μm for TL. A lower level of preserved DNA integrity was shown by the original species, i.e. blue foxes (B) and silver foxes (S), for which the analysed parameters were similar and the tail DNA was equal to 24.98±20.85% in B and 25.72±20.58% in S (Table 2). For individuals of both species, HD ranged from 74.28±20.58% (S) to 74.93±20.96% (B) and TL from 142.09±88.03 μm (S) to 151.67±97.25 μm (B). For H vs. B and S, the interaction between sex and species was significant ($p<0.05$) for head DNA, tail DNA and OTM, and highly significant ($p<0.01$) for tail length and TM.

Additionally, TM and OTM values were determined for all three fox species (H, B and S). Likewise, hybrids differed from blue and silver foxes. Detailed data are provided in Table 2.

The comets localized on the preparations were evaluated for tail length and later classified according to GEDIK *et al.* (1992). This method scores comets visually according to DNA damage and classifies cells into 5 categories. The first class (N) includes intact lymphocytes or those with less than 5% damage. The second (L) covers cells with nuclear DNA fragmentation ranging from 5% to 25%. The third class encompasses cells with DNA disintegrity of 25-40% (M) and 40-95% in the comet tail (H). The last class (T) includes heavily damaged cells with over 95% DNA damage in the tail, known as hedgehog comet (GEDIK *et al.* 1992). Gedik's scale allowed us to make a quick estimate of the results. The cells of the blue and silver foxes fell within the L class (5-25% DNA in the comet tail) (Figure 1). Most of the cells analysed in the hybrids were classified as N with less than 5% DNA damage in the comet tail. The results are presented graphically in Figure 1a-c.

Table 1

Parameters of DNA integrity in lymphocytes in sex

Sex[1)]	% Head DNA		% Tail DNA		Tail length (μm)		TM		OTM	
	Mean	SD[2)]	Mean	SD[2)]	Mean	SD[2)]	Mean	SD[2)]	Mean	SD[2)]
F	79.84[A]	18.19	20.09[A]	18.06	115.08[A]	86.91	35.56[A]	53.22	21.41[A]	30.04
M	77.54[B]	21.32	22.46[B]	21.32	126.63[B]	104.11	47.81[B]	73.25	27.46[B]	41.43

[1)] Sex: F – Female, M – Male;
[2)] SD – Standard Deviation;
[A, B] Values within the same column marked with different letters differ highly significantly ($p<0.01$).

Table 2

Parameters of DNA integrity in lymphocytes in hybrids, blue and silver fox

Species[1)]	Head %DNA		Tail %DNA		Tail length (μm)		TM		OTM	
	Mean	SD[2)]	Mean	SD[2)]	Mean	SD[2)]	Mean	SD[2)]	Mean	SD[2)]
H	91.69[A]	5.98	8.31[A]	5.98	34.81[A]	24.09	4.03[A]	10.47	2.72[A]	6.99
B	74.93[B]	20.96	24.98[B]	20.85	151.67[B]	97.25	53.80[B]	70.48	32.04[B]	40.02
S	74.28[B]	20.58	25.72[B]	20.58	142.09[B]	88.03	52.20[B]	66.28	29.72[B]	36.61

[1)] Species: H – interspecific hybrids, B – blue fox, S – silver fox;
[2)] SD – Standard Deviation;
[A, B] Values within the same column marked with different letters differ highly significantly ($p<0.01$)

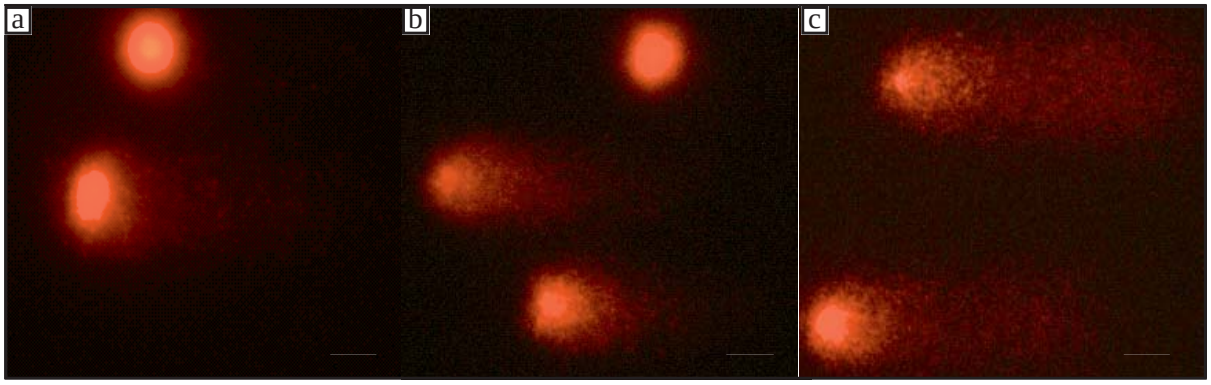

Fig. 1. Lymphocytes of interspecific hybrids – a, blue foxes – b, and silver foxes – c with different levels of DNA damage in the comet assay. 400x magnification. Bar = 10 μm.

Discussion

Our study showed that chromosomal polymorphism has no effect on DNA integrity in the interspecific hybrids of the blue and silver foxes. The chromosomal polymorphism in the hybrids included a variable number of A-chromosomes, with modal diploid number 2n=40, 41 or 42, and number of B-chromosomes ranging from 0 to 4 (BUGNO-PONIEWIERSKA *et al.* 2015; SZELESZCZUK *et al.* 2018). It was observed, however, that the presence of one type of chromosomal polymorphism in the blue and silver foxes reduces DNA stability in the somatic cells of these species. Sex was found to influence DNA sensitivity in the studied animals. Based on the results the differences between sex could be connected with a higher number of chromosomal polymorphisms in male cells which slightly reduce DNA stability. Research on genomic instability in foxes (GRZESIAKOWSKA *et al*. 2017; SZELESZCZUK *et al*. 2018) revealed a lower amount of two types of chromosomal instability in male cells as sister chromatid exchange and the presence of micronuclei in blue and silver fox. This shows that male cells are more stable than female cells. In the case of hybrids, a higher average percentage of binucleated cells with micronuclei was observed in cells of males, but not female.

Large differences were noted for the effect of species on DNA stability. In each case, the hybrids were more resistant to endogenous damage. The cells of hybrids were characterized by higher genetic stability in comparison to blue and silver fox. This could be related to the presence of both types of karyotype polymorphism in cells of hybrids. In this case, their genetic material during interphase might be more stable and resistant to fragmentation and DNA strand breaks as shown in this study. Opposite results were obtained by SZELESZCZUK *et al.* (2018) who investigated damage occurring during cell division. The hybrids were characterized by increased chromosomal instability observed as the presence of micronuclei in binucleated cells. Both fox species, blue and silver, were characterized by a lower amount of damage in the micronucleus test (SZELESZCZUK *et al.* 2018). This might be connected with higher sensitivity of hybrid cells during division, which could be caused by chromosomal instabilities such as the presence of micronuclei or sister chromatid exchange.

In preliminary research using comet assay, GRZESIAKOWSKA and SZELESZCZUK (2017) observed reduced stability of genomic DNA in somatic cells of blue and silver fox. Based on two parameters: the percentage of head DNA and the tail length, higher DNA sensitivity to damage was found in the case of blue fox. In the case of chromosomal instability, especially sister chromatid exchange and the presence of micronuclei, the blue fox cells were more stable in comparison to silver fox (GRZESIAKOWSKA *et al.* 2017; SZELESZCZUK *et al.* 2018). This might suggest that the presence of centric fusions as karyotype polymorphism is a slightly more stable form than additional B-chromosomes during cell division.

Chemical modifications of chromatin, caused by exogenous or endogenous genotoxic factors, disrupt chromatin structure. This instability generates further mutations, which may lead to malformations, embryo death and reproductive disorders, and cause neoplastic transformation (ASHBY 1995; LIPTÓI *et al.* 2005; MØLLER 2006; WÓJCIK and SMALEC 2012, 2013). Detection of DNA damage at a single-cell level is of prime importance in fields such as genetic toxicology (DNA damage in cells *in vitro* and *in vivo*), pharmaceutics, monitoring of DNA repair after chemo- and radiotherapy, ecotoxicology, animal and human nutrition, biomonitoring of genotoxicity, epidemiology, evaluation of material deposited in semen banks, toxicology, and diagnosis of genetic diseases. In addition, this analysis is used in tests and in envi-

ronmental biomonitoring (ANDERSON *et al.* 1998; TICE *et al.* 2000;, COLLINS *et al.* 2001; FAUST *et al.* 2004; ISAKSSON 2010).

The comet assay is a sensitive technique for measuring and repairing DNA damage at the level of single cells. This versatile test evaluates damage in different types of cells and samples of both peripheral blood and culture cells, cancer cells, solid tumors, semen, yeast, and bacteria (AWAD *et al.* 2014; CZUBASZEK *et al.* 2014). In our study, lymphocytes isolated from whole peripheral blood were studied.

Single cell gel electrophoresis (SCGE) is a method for electrophoretic separation of nuclear DNA enabling observation of DNA fragmentation. Migration capacity in the electric field is associated with the negative charge of DNA strands and DNA fragments. The examined cells are immobilized in agarose on microscope slides and lysed to release their contents. The high ionic strength of the lysis buffer increases the dissociation of proteins from DNA. This is followed by electrophoresis. DNA is stained fluorescently or with silver salts, and the obtained image is analysed under a microscope. The image resembles a comet if damage occurs. The comet head is the area covered by the cell nucleus, and the comet tail is made up of DNA fragments that migrated during electrophoresis. The greater the damage to the cell nucleus, the longer the tail of the comet. The level of DNA damage is measured by the length of the tail and the amount of DNA it contains. A reliable result is obtained based on analysis of 50-100 comets in a gel (SINGH *et al.* 2003; KOŃCA *et al.* 2003). In our study, the parameters used to evaluate the extent of DNA damage in the SCGE alkaline assay were HD, TD and TL as well as TM and OTM values. The Tail Moment (TM) is calculated as the product of the tail length and the percentage of DNA in the tail. Olive Tail Moment (OTM) is defined as the product of the % DNA in the tail multiplied by the distance between fluorescence intensity in the center of gravity in the head and the center of gravity in the tail along the comet X-axis (COLLINS *et al.* 2008; KUMARAVEL *et al.* 2009). The purpose of using several evaluated parameters was to select the most useful one. Our study suggests that individual variation within the examined traits was at a similar level for each parameter. This shows that each parameter can be used interchangeably in experiments that use the SCGE assay. In addition, we used 50 cells from each individual. This number of observations was representative and produced a reliable result comparable between the individuals.

The alkaline comet assay is most commonly used, but a neutral version of the comet assay is also available (NANDHAKUMAR *et al.* 2011). The difference is essential because the alkaline SCGE assay is able to detect both single- and double-strand DNA damage. The method has the advantage of analysing the extent of several different DNA modifications with relatively small changes in the basic test procedure. The method identifies single- and double-strand breaks of the DNA as well as all chemical (apurinic sites, unstable adducts) and enzymatic modifications (oxidative damage), which may potentially develop into breaks. The comet assay detects DNA damage at the single-cell level, and determines sensitivity to specific genotoxic factors, as well as DNA repair efficiency (ASHBY *et al*. 1995; SINGH *et al.* 1988). The assay may analyse any tissues from which a cell suspension is obtained.

In future studies, it is worth considering changes in the genetic material of cells of more age-differentiated animals (young and old) and animals breeding in different areas. It would be useful for animal breeding to check the degree of DNA damage in semen cells of males used for reproduction.

The results obtained and presented in this study, as well as future research, are an important element in monitoring the condition of breeding animals' health. Changes occurring within the genetic material may indicate serious disease processes of individual units. However, in studies involving a group of healthy farm animals, observed changes in DNA integrity may indicate external factors. And such monitoring seems important also for human health. In the case of farm animals, an important factor that may somehow affect the general condition of the organisms is that they are fed by animal by-products from the food industry. These products are considered to be environmentally hazardous and should not be co-deposited or disposed of with other methods. The cheapest alternative is to convert them into feed for farm animals. However, there is a risk of accumulation of harmful compounds in these products that may adversely affect the condition of the animals. The proposed test method can allow monitoring the health status of animals.

In our study, we used the alkaline SCGE assay. Each examined parameter made it possible to evaluate the stability of genetic material in the somatic cells of the studied species. The obtained results provide conclusive evidence that the chromosomal polymorphism in the cells of the original species (blue and silver foxes) does not adversely affect the genetic stability of interspecific fox hybrids.

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Author Contributions

Research concept and design: A.G., O.S.; Collection and/or assembly of data: A.G.; Data analysis and interpretation: A.G., O.S., M.K-G., A.O-M.; Writing the article: A.G., M.K-G.; Critical revision of the article: M.K-G.; Final approval of article: O.S., A.O-M.

Conflict of Interest

The authors declare no conflict of interest.

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