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Analysis of genomic Instability in Primary Spermatocytes of Interspecific Hybrids of the Red Fox (*Vulpes vulpes*) and the Arctic Fox (*Alopex lagopus*)*

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The aim of this study was to analyse meiotic cells of male interspecific hybrids of the red fox (*Vulpes vulpes*) and the arctic fox (*Alopex lagopus*). To this end we determined stages of meiotic cells as well as carried out FISH analyses with probes specific to heterosomes and a TUNEL assay on synaptonemal complex preparations. The meiotic cell analysis revealed only the presence of stages of the first meiotic division from leptotene to pachytene. Moreover, we observed an increased level of early dissociation of the X-Y bivalent as well as a high percentage of apoptotic cells. These results indicate the disruption of meiotic division in male hybrids manifested through meiotic arrest of the cells. Faulty pairing of the heterosomes can be considered as one of the causes leading to the initiation of the apoptotic process.

Key words: *Alopex-Vulpes* hybrids, heterosomes, interspecific hybrids, meiosis, synaptonemal complexes.

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Interspecific hybrids of the red fox (*Vulpes vulpes* L. – *V. fulva* species) and the arctic fox (*Alopex lagopus* L.) are of great value to breeders because of improved production traits such as health or fur as well as a larger number of offspring in a litter than in the red fox (GUSTAVSSON *et al*. 1988). On the other hand, the hybrids are sterile which is caused by the different number and morphology of chromosomes of the parental species (GRAPHODATSKY & RADZHABLI 1981; MAKINEN & GUS- TAVSSON 1982). In the arctic fox, three variants of the karyotype resulting from differential occurrence of a centric fusion can be present: 2n=48, 2n=49 or 2n=50 (GUSTAVSSON & SUNDT 1965). Conversely, the red fox is characterized by a fixed number of A chromosomes, which is 2n=34, however, it possesses a variable number of B chromosomes ranging from 0 to 8 (GRAPHODATSKY *et al*. 2000). These differences in the number of chromosomes lead to the creation of an atypical, unbalanced karyotype of hybrids which in consequence causes multivalent formation followed by disruption of meiosis process (GUSTAVSSON *et al*. 1988). Consistently, disturbances in gametogenesis may inhibit the gametogenic process and make animals infertile. In general, studies on the course of meiosis are focused on the following division stages: prophase I pachytene (chromosome pairing), metaphase I (chiasma formation and terminalisation) and metaphase II (segregation of homologous chromosomes) (ŚWITOŃSKI & STRANZINGER 1998).

Meiosis analyses can be carried out with the use of conventional techniques based on cell hypo-

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tonization, Carnoy fixation and Giemsa staining, as well as molecular techniques based on hybridization of a DNA probe with a homologous chromosome region. The latter methods, such as FISH (fluorescence *in situ* hybridization), are recommended for analysing various aspects of meiosis, i.a. the recombination process, as well as for analysing disruptions of this division by virtue of highly accurate chromosome identification (FROENICKE *et al.* 2002; CODINA-PASCUAL *et al.* 2004; VILLAGÓMEZ & PINTON 2008). Thus, the use of FISH technique with probes specific to heterosomes allows for observations of patterns and abnormalities occurring at different stages of meiotic division. Additionally, in order to assess disruptions of physiological state of a cell, TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, which allows for visualization of apoptotic cells, can be employed.

Material and Methods

The foxes were kept and slaughtered on a farm. The research was carried out under the project No N N311 542540 and has permission of the II Local Ethics Committee in Kraków (no 771, 22nd July 2010). The testes were collected postmortem from 15 *Alopex-Vulpes* male hybrids. Horse material was obtained from a routine breeding procedure – the castration of males under the age of 3 years which had not been selected for further breeding (the material constitutes "surgical waste" and does not require the approval of the Ethics Committee).

Meiotic preparations

Testicular samples were placed onto a watch glass and ground into small pieces. The obtained homogenate was transferred to a hypotonic solution (1% sodium citrate) and shaken for 2h, collecting 1 ml at 15-min intervals. The collected samples (8 x per test tube) were centrifuged for 8 min (1500 rpm). The supernatant was then removed and the pellet was fixed in a mixture of methanol and ice-cold acetic acid (3:1). This procedure was repeated six times. The suspension was dropped onto a slide, the preparations were air dried and stained with 5% Giemsa for 20 min. The preparations were observed under an Opton Axiophot fluorescence microscope and successive stages of meiosis were saved on a computer using Lucia software.

Synaptonemal complex preparations

In order to obtain synaptonemal complex preparations, we carried out testicular tissue maceration. Then, the cell suspension was placed on a drop of 0.2 N sucrose on a glass slide which was followed by fixation with 4% paraformaldehyde in 0.2 N sucrose for 10 min and rapid washing in detergent solution (0.4% Photo-flo, Kodak). Next, the preparations were dried in a vertical position. Selected preparations were stored at -20°C until further analysis.

FISH technique

Molecular probes specific for the dog X and Y sex chromosomes suspended in hybridization mix (50% formamide, 10% dextran sulphate, 10% 20xSSC, 1% Tween 20 and 29% H_2O) were applied on to the synaptonemal complex preparations. The probes were obtained with the use of manual microdissection of the dog heterosomes. Briefly, the preparations were denatured in 70% formamide in saline-sodiumcitrate buffer (2xSSC) for 2.5 min at 70°C. The probes were denatured at 70°C for 10min. The hybridization was carried out in 37°C for two days. Post-hybridization washes were as follows: three times with 50% formamide in 2xSSC and three times in 2xSSC at 42°C. The X probe was labeled with biotin so hybridization signals were detected by the avidin-FITC (fluorescein isothiocyanate) and anti-avidin system. The Y probe was labeled with Cy3 which does not need additional detection. The preparations were then mounted with DAPI stain in a mounting medium. Microscopic evaluation was performed under an Opton Axiophot fluorescence microscope equipped with a camera and Lucia software. We classified chromosomes as dissociated when they were distant from each other at least at a distance corresponding to the size of the Y chromosome.

TUNEL assay

To assess DNA fragmentation resulting from apoptosis, synaptonemal complex preparations from seven hybrids and three horses (control) were subjected to TUNEL assay using the In Situ Cell Death Detection Kit, Fluorescein (Roche) along with protocol-specified controls for both species. The horses were fertile, did not have any chromosomal abnormalities and were castrated only because of breeding selection. First, the preparations were rinsed in PBS for 5 minutes. Then, they were incubated in permeabilization solution for 2 min, on ice, according to the manufacturer's protocol. Afterwards, the preparations were rinsed 2x5 min in PBS. Next, 50 μl of a mix of enzyme and label solutions were added to each slide and covered with coverslips. The slides were incubated in a humidified chamber in the dark in 37°C for 60 min. After incubation, the preparations were rinsed 3x5 min in PBS, air dried and mounted with DAPI

stain in a mounting medium. Microscopic evaluation was carried out under an Opton Axiophot fluorescence microscope equipped with a camera and Lucia software. To estimate the significance of the TUNEL results, we used R software to employ the Shapiro-Wilk test for normality and then Student's *t*-test for unpaired samples.

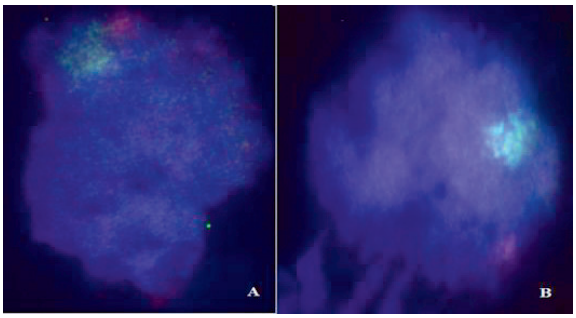

Fig. 1. FISH technique with probes specific to the whole X and Y chromosomes on synaptonemal complex preparations of the hybrids. The X chromosome signal is green, the Y chromosome signal is red. A – a cell with associated X-Y bivalent, B – a cell with dissociated X-Y bivalent.

Results

Meiotic preparations

Meiotic slides prepared from testicular tissue of 15 *Alopex-Vulpes* male hybrids were analysed under a light microscope to determine the successive stages of meiotic division. We observed only stages of the first meiotic division, from leptotene to pachytene. No further stages were observed.

FISH technique

Fluorescence *in situ* hybridization with probes specific to the X and Y chromosomes was used to visualize heterosomes and analyse their pairing in synaptonemal complexes. The X chromosome was indirectly stained with biotin giving a green signal, and the Y chromosome was directly stained with cyanine 3 (Cy3) giving a red signal (Fig. 1). A total of 2227 cells were analyzed, with an average of 148 cells per animal. Of the analyzed cells, 45% had associated heterosome bivalents and 41% had them dissociated. Moreover, 11% of the counted cells had only the X chromosome and 3% only the Y chromosome. The percentage of dissociated bivalents ranged from 30% to 70%, whereas the percentage of X and Y monosomy ranged from 2% to 20% and from 1% to 8%, respectively. More detailed data are presented in Table 1.

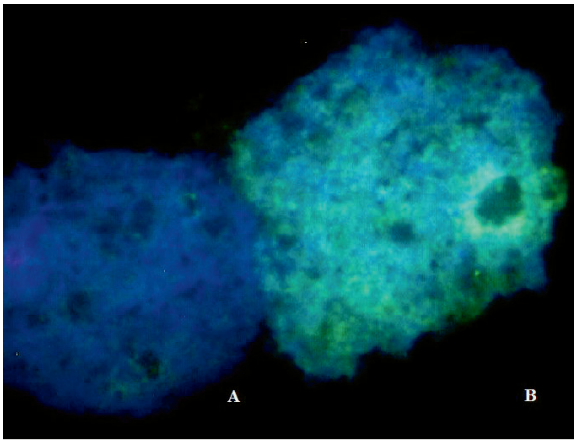

Fig. 2. TUNEL assay carried out on synaptonemal complex preparations. Green, fluorescent colour indicates apoptosis. A – a non-apoptotic cell, B – an apoptotic cell.

TUNEL assay

To estimate the percentage of cells undergoing apoptosis, TUNEL assay was carried out on synaptonemal complex preparations. Cells were classified as apoptotic if they exhibited a distinct, green, fluorescent signal (Fig. 2). At least 100 cells of each preparation were analyzed, giving in total 1875 cells. The percentage of apoptotic cells ranged from 17 to 43 for the hybrids (31% on average), while for the control it amounted to 7% on average. More detailed data are presented in Table 2. After confirmation of normality of the data, we employed Student's t-test which revealed highly significant ($P<0.001$) differences between the analyzed and control groups (Figure 3).

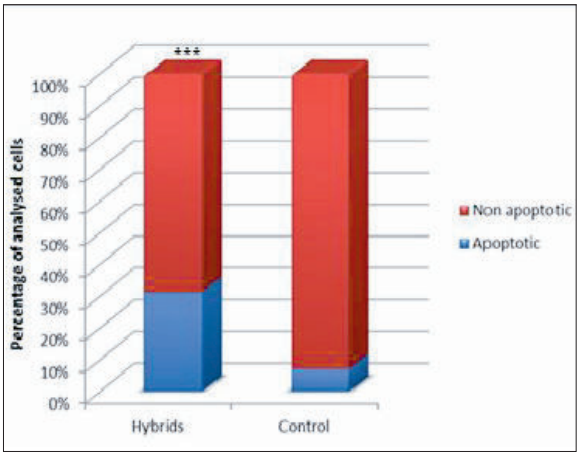

Fig. 3. Percentage of apoptotic and non-apoptotic cells in the hybrids and controls, detected by the TUNEL assay ($P<0.001$).

Table 1

The results of the analysis of the X-Y bivalent pairing in the synaptonemal complexes with the use of the FISH technique

Animal no.	Cells with associated heterosomes	Cells with dissociated heterosomes	Cells with only the X chromosome	Cells with only the Y chromosome	Sum
1	130	117	-	–	247
2	66	66	28	1	161
3	76	74	30	9	189
4	55	62	18	8	143
5	55	71	10	3	139
6	57	47	27	8	139
7	96	75	50	20	241
8	87	56	29	10	182
9	53	82	23	13	171
10	77	52	10	–	139
11	84	51	4	–	139
12	53	50	-	–	103
13	50	51	2	1	104
14	60	44	2	–	106
15	7	17	–	–	24
∑	1006 (45%)	915 (41%)	233 (11%)	73 (3%)	2227

Table 2

The results of the analysis of apoptosis carried out on synaptonemal complex preparations of the hybrids and controls with the use of the TUNEL assay

Animal no.	Apoptotic cells (%)	Non apoptotic cells (%)	Sum
1	40 (20%)	161 (80%)	201
2	88 (43%)	116 (57%)	204
3	71 (30%)	159 (70%)	230
4	37 (18%)	170 (82%)	207
5	63 (31%)	140 (69%)	203
6	87 (42%)	122 (58%)	209
7	71 (34%)	135 (66%)	206
SUM	457 (31%)	1003 (69%)	1460
Control 1	13 (11%)	109 (89%)	122
Control 2	3 (2%)	138 (98%)	141
Control 3	13 (9%)	139 (91%)	152
∑	29 (7%)	386 (93%)	415

Discussion

In the present study, we observed meiotic stages in cells obtained from testicular tissue of interspecies hybrids of the red fox and the arctic fox. Analysis of all preparations revealed only cells in the early meiotic stages, from leptotene to pachytene, suggesting a disruption of meiotic division which is in concordance with other studies (GUSTAVSSON *et al.* 1988; NYBERG 1980; WIPF & SHACKELFORD 1949). Considering that hybrids are carriers of an unbalanced karyotype, this meiotic arrest prevents the cells from further division. This from further division which could lead to the creation of unbalanced gametes, which is why, in consequence, the hybrids are sterile. This mechanism has been reported in other species and other types of karyotype aberrations (KOYKUL *et al.* 2000; FOREJT 1979), however some exceptions have also been reported (GROPP *et al.* 1970; MOSES *et al.* 1979). To further investigate the nature of this disruption, we carried out an analysis of the X-Y biva-

lent pairing in the synaptonemal complex preparations with the use of FISH with probes specific for the X and Y chromosomes. This analysis can provide useful information on the course of the disrupted meiosis because the X-Y bivalent exhibits different behaviour in particular substages of prophase I. Alterations of the pattern of behaviour may be highly informative in terms of possible causes of meiotic arrest. In general, during late zygotene autosomal chromosomes are separate and the X and Y chromosomes start pairing in terminal segments. Then, in early pachytene, autosomes begin to pair and the X and Y chromosomes continue pairing till mid-pachytene when all chromosomes (both autosomes and heterosomes) are tightly paired. Finally, during late pachytene autosomes continue to pair and the X-Y bivalent begins to dissociate (VILLAGÓMEZ 1993). In our study we observed that 41% of the analyzed cells had the X-Y bivalent dissociated, 11% had only the X chromosome and 3% only the Y chromosome. These results suggest that the course of meiosis is disrupted in hybrids, manifested as either early dissociation of the bivalent, a failure of the bivalent to pair or the loss of one of the sex chromosomes. This in consequence may lead to the observed meiotic arrest and sterility (MATSUDA *et al.* 1991). Similar studies on the pairing of the X-Y bivalent of the red fox and the Chinese raccoon dog were conducted by SOSNOWSKI and co-authors (2011). They showed that the percentage of the dissociated bivalent amounted to 15.6% for the raccoon dog and 0.2% for the red fox. Their results, despite the relatively high ratio of dissociation for the raccoon dog, are discordant with our findings. The discrepancies may be partially caused by inter-species differences but mainly by the disruption of the meiotic division of the hybrids.

Furthermore, inter-individual diversity of the percentage of the disrupted cells as well as the lack of predominance of these cells indicate the presence of another concurrent mechanism responsible for hybrid sterility. Given that the hybrids bear an unbalanced karyotype, the autosomal nature of the disrupted meiosis should also be considered. This assumption remains in concordance with our results indicating meiotic arrest of hybrid cells because distorted pairing of autosomal chromosomes may also inhibit meiotic division (GUSTAVSSON *et al.* 1988).

In order to give insight into processes taking place within meiotic cells of the hybrids, we performed TUNEL assay which visualizes cells undergoing apoptosis. Our results suggest that this process can be connected with meiotic arrest because we observed a high percentage of apoptotic cells on the hybrid preparations and the differences between the hybrids and the controls were statistically highly significant. Initially, there were concerns that TUNEL assay may not be specific only for apoptosis, because, due to its ability to label DNA breaks, it can detect necrosis and strand breaks characteristic for early meiotic processes (GRASL-KRAUPP *et al*. 1995) thus generating false-positive results. However, current TUNEL protocols applying pretreatment procedures have substantially increased sensitivity to apoptosis and thus they preferentially detect cells undergoing this process (GOLD *et al.* 1994; GORCZYCA *et al.* 1993; NEGOESCU *et al*. 1996, 1998). As a control, we used SC preparations prepared from horse testicular tissue of sexually mature animals, due to the lack of red and arctic fox preparations. Because the karyotype of the horses was normal, we were able to assess the level of apoptosis in cells without chromosomal rearrangements, providing information about the level of apoptosis caused by chromosomal rearrangements in the hybrids. However, we cannot exclude the influence of species differences. Altogether, these results indicate that apoptosis may be a part of a mechanism leading to the elimination of cells in meiotic arrest. Similar observations concerning X-autosome translocation carrier boars were made by KOYKUL and co-authors (2000). However, the level of apoptotic cells could be expected to be higher if we take into consideration the absence of stages beyond pachytene, the high ratio of X-Y bivalent aberrations and an unbalanced karyotype. Nevertheless, we suggest that the observed percentage of apoptotic cells may result from the fact that not all cells undergo apoptosis at the same time, i.e. at the moment of detection.

In conclusion, meiotic division of the male hybrids of the red fox and the arctic fox is disrupted. The disruption is manifested through the meiotic arrest of cells at the pachytene stage, leading to the sterility of the animals. Our research suggests that lack of X-Y pairing as well as loss of one of the sex chromosomes are largely responsible for the meiotic arrest. Moreover, as a consequence of the cease of the meiotic process, the cells are eliminated through apoptosis. Nevertheless, because the karyotype of the hybrids is unbalanced, aberrations of autosomal segregation are also considered as another cause of meiotic disruption.

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