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Heterosome X premutation structural changes associated with fertility of Romanov sheep*

Przedmutacyjne zmiany struktury chromosomu X związane z płodnością owcy romanowskiej

Summary. Cytomolecular analysis of spontaneous and *in vitro* induced heterosome X permutation structural changes in Romanov sheep revealed a high level of Xq11, Xq22, Xq31 and Xq32/33 region instability in infertile ewes. The highest frequency of structural defects was found in OAR Xq22 region comprising unstable trinucleotide CGG repetitions of *FRM1* gene regulatory sequence, identified by *in situ* PCR method. The results obtained suggest that chromosome X structural instability in sheep may be the cause of inappropriate expression of genes determining reproductive traits and increased rate of aneuploid germ cells related to prenatal and perinatal mortality.

Key words: sheep, heterosome X instability, fragile X, *FRM1* gene, trinucleotide tandem repetitions

INTRODUCTION

Structural chromosome instability is considered to reflect DNA damages, which, if not repaired, result in gene or chromosome mutations. Exposure to environmental genotoxic agents was found to increase frequency of spontaneous unstable karyotype lesions, preferentially affecting some chromosomes or their regions [Bryant 1997]. Specific in vitro lymphocyte culture conditions or chemical treatment with mutagens or clastogens (e.g. APC – aphidicolin: DNA polymerase inhibitor or 5-AZA/BrdU – 5-azacytidine/bromodeoxyuridine – DNA nucleosides substitution agents) were also

proved to cause a statistically significant recurrence of structural defects (breaks/gaps) at the same chromosome points, regarded as fragile sites [Sutherland *et. al*. 1985, 1996]. Chromosomal fragility is considered to play a role in karyotype evolution, chromosomal rearrangements and disease etiology related to productive and reproductive efficiency of farm animals [Danielak-Czech and Słota 2004, Usdin 2008]. Thus, extensive studies have been undertaken on the fragile sites (FS) (nonrandom chromosomal breaks/gaps) in several species of *Bovidae* regarding different methods of induction, and their clinical and biological significance [Riggs and Rønne 2009]. In result, bovine chromosome fragility (mainly chromosome X) were revealed to be associated with pathologies (baldy calf syndrome, dwarfizm) and fertility impairment (repeat breeders, long calving interval, abortions) [Uchida *et al*. 1986, Llambi and Postiglioni 1997, Rincón *et al*. 1997, Słota *et al*. 2000, Danielak-Czech and Słota 2002, Słota and Danielak-Czech 2002]. Moreover, as with fragile X in humans, some latest studies in *Bovids* attempt to link this phenomenon with structurally unstable chromosome X *loci* containing tandem repeated sequences, e.g. trinucleotide CGG repeats of the *FRM1* gene, which were mapped to BTA Xp13 and OAR/CHI Xq22 regions [Danielak-Czech and Słota 2006, Słota *et al*. 2007a, b, Kaczor *et al*. 2009]. In sheep, both autosomal and chromosome X-specific fragile sites have been studied sporadically till now, and their clinical effects still remain to be determined [Cribiu *et al*. 1991, Matejka *et al*. 1995, Danielak-Czech and Słota 2002, 2004, Słota and Danielak-Czech 2002, Ahmad *et al*. 2008, Danielak-Czech *et al*. 2010].

The aim of the present study was to evaluate spontaneous as well as *in vitro* induced chromosome X structural defects in subfertile ewes of Romanov sheep.

MATERIAL AND METHODS

Cytogenetic evaluation was performed in population of 24. exterior normal, 2–5 years ewes of Romanov sheep, taking into account individual reproductive efficiency. The experimental group (12 sheep) included infertile ewes owing to ineffective matings (100% in two years) and the control group (12 sheep) was composed of ewes having normal reproductive performance.

Slides of sheep metaphase chromosome were prepared following classical cytogenetic protocols of lymphocyte culture (both untreated and exposed to 0.4 μM APC or 0.1 μM FudR 24 h before harvesting) and banding techniques (GTG/QFQ/DAPI). Karyotypes were arranged according to the sheep international karyotype standards [Di Berardino *et al*. 1990, 2001].

The pair of primers: 5’ GCTCAGCTCGGTTTCGGTTTCACTTCCCGT 3’ (forward) and 5’ AGCCCCGCACTTCCACCACCAGCTCCTCCA 3’ (reverse) flanking CGG repeats of the 5’UTR region of human *FRM1* gene (0.3830 kb) (GDB: 187391; c/f) [Fu *et al*. 1991] were used for *in situ* PCR and biotin-16 dUTP labeling [Troyer *et al*. 1994] of the homologous sequence directly on microscopic slides with metaphase chromosome spreads (in MJR PTC-100 thermocycler with metal heating block for glass slides). The reaction was carried out according to the profile: 1 cycle: 94°C – 3 min, 65°C – 1 min, 72°C – 1 min; 30 cycles: 94°C – 1 min, 65°C – 1 min, 72°C – 1 min. The amplified and labeled gene fragment was detected by avidin-conjugated FITC, and hy-

bridization signals were analyzed with fluorescence microscope equipped with the computer-assisted image analysis system LUCIA-FISH (Czech Republic).

The results were estimated using analysis of variance, Chi square test.

RESULTS AND DISCUSSION

The results of cytogenetic evaluation carried out in the experimental and control groups of ewes are given in Table 1, which includes the mean total frequency (%) of spontaneous and *in vitro* induced breaks/gaps in karyotype and separately in the pair of X heterosomes, calculated for each group and compared statistically between groups.

Table 1. Frequency of spontaneous and *in vitro* induced chromosome structural defects in groups of fertile and infertile ewes

Tabela 1. Częstość spontanicznych i indukowanych *in vitro* strukturalnych defektów chromosomów w grupach płodnych i niepłodnych maciorek

Group n = 12 Grupa n = 12	Spontaneous chromatid breaks/gaps Spontaniczne pęknięcia/przewężenia chromatyd		APC – induced chromatid breaks/gap Pęknięcia/przewężenia chromatyd indukowane *in vitro* APC		5-AZA- and BrdU – induced chromatid breaks/gaps Pęknięcia/przewężenia chromatyd indukowane *in vitro* 5-AZA- I BrdU	
	total % SD ogółem % SD	X chromosome % chromosom X %	total % SD ogółem % SD	X chromosome % chromosom X %	total % SD ogółem % SD	X chromosome % chromosom X %
e.	2.88 ±2.10	1.59	44.04* ±11.05	17.12*	12.66 ±4.99	6.33*
c.	1.96 ±1.02	1.11	30.99 ±10.10	10.25	9.74 ±2.89	3.91

e. – the experimental group – grupa eksperymentalna, c. – the control group – grupa kontrolna, * significant differences ($P < 0.05$) – różnice istotne ($P < 0,05$)

Table 2. Frequency of *in vitro* induced structural defects at unstable chromosome X regions in groups of fertile and infertile ewes

Tabela 2. Częstość indukowanych *in vitro* defektów strukturalnych w niestabilnych regionach chromosomu X w grupach płodnych i niepłodnych maciorek

Chromosome X unstable region Niestabilny region chromosomu X	Chromatid breaks/gaps (%) Pęknięcia/przewężenia chromatyd (%)	
	experimental group grupa eksperymentalna	control group grupa kontrolna
q11 (5-AZA- and BrdU sensitive)	2.16 ±1.89	1.85 ±1.11
q22 (APC – sensitive)	9.88** ±6.95	5.29 ±2.30
q31 (APC – sensitive)	5.20* ±2.07	3.05 ±1.99
q32/33 (5-AZA- and BrdU sensitive)	2.78* ±2.51	1.24 ±1.06

* significant differences ($P < 0.05$) – różnice istotne ($P < 0,05$)

** highly significant differences ($P < 0.01$) – różnice wysoko istotne ($P < 0,01$)

As shown in Table. 1, the total frequencies of APC-sensitive breaks/gaps in the groups studied exceeded of 5-AZA- and BrdU–sensitive damages. Generally, percentages of X-specific lesions were significantly higher in the experimental group comparing to the control one. The frequencies of breaks/gaps at excessively damaged Xq31, Xq22 and Xq32/33 regions, given in Table 2 and presented in Figure 1, differed on the statistically significant or highly significant level between groups.

The highest frequency of structural defects was found in karyotype of infertile ewes in OAR Xq22 region comprising unstable trinucleotide CGG repeats of the *FRM1* gene, which were mapped basing on combined *in situ* PCR method and GTG/QFQ/DAPI banding techniques (Fig. 1 C).

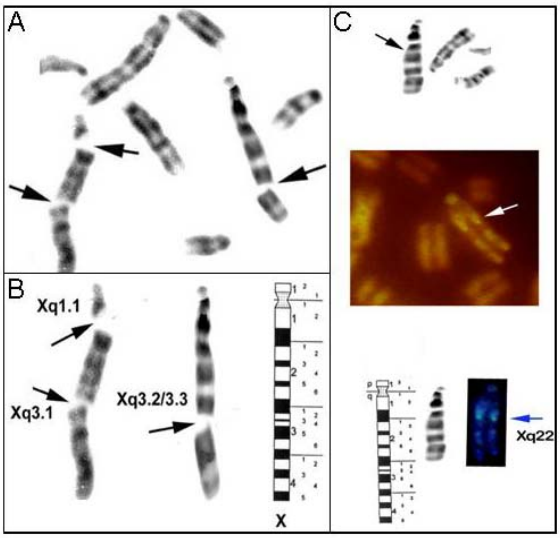

Fig. 1. Fragile sites of chromosome X in infertile ewes: OAR Xq11, Xq31, Xq32/33 (A, B) and OAR Xq22 comprising unstable trinucleotide CGG repeats of *FRM1* gene regulatory sequence identified by *in situ* PCR method (C)

Ryc. 1. Miejsca łamliwe chromosomu X u niepłodnych maciorek: OAR Xq11, Xq31, Xq32/33 (A, B) i OAR Xq22 zawierające niestabilne trzynukleotydowe powtórzenia CGG sekwencji regulatorowej genu *FRM1* zidentyfikowane metodą *in situ* PCR (C)

The BrdU-inducible fragile sites in the Romanov and Booroola sheep karyotype have been reported to be located at the autosomal subcentromeric bands of the 9 as well as presumptive 12 and 20 chromosome pairs, respectively [Cribiu *et al*. 1991, Matejka *et al*. 1995]. However, the 9q1.1 fragile site carriers were stated to have some reproductive parameters (ovulation rate, litter size, embryonic mortality) on the level similar to control animals. On the other hand, the present study carried out with the Romanov sheep revealed 5-AZA- and BrdU-dependent Xq1.1 and Xq3.2/3.3 region fragility, especially distinct in the group of ewes repeating ineffective matings. Moreover, the Xq22 and Xq31 bands have been found to be the extremely expressive APC-induced fragile sites in infertile ewes of this breed. On the basis of the results obtained, the Xq3.2/3.3 5-AZA-

and BrdU-sensitive bands were found to correspond to Xq3.3 folate-dependent site as well as to adjoin the Xq3.1 APC-induced one which were identified previously in the group of subfertile ewes of the old native Polish Heath sheep [Słota and Danielak-Czech 2002, Danielak-Czech and Słota 2002, 2004, Danielak-Czech *et al*. 2010]. Interestingly, the sheep Xq31-33 chromosome region (including mentioned above Xq32/33) is known to demonstrate synteny conservation and segment homology with the fragile cattle Xq26-31chromosome segment, observable in some cases in the repeat breeding cows [Di Berardino *et al*. 1983, Iannuzzi and Di Meo 1995, Prakash *et al*. 1997].

Our findings have proven that adequate sheep/cattle unstable chromosome regions represent common fragile sites preserved in species of *Bovidae*, which may accelerate revealing of candidate genes for fertility impairment associated to chromosomal fragility in this family. For example, the trophinin gene (TRO) responsible for bovine embryo implantation, which has been mapped to fragile Xq25-33 region conserved in family *Bovidae*, can be supposed to affect sheep reproduction [Asai *et al*. 2004]. Alike, the excessive fragility of OAR Xq22 region with trinucleotide CGG repeats of *FRM1* gene, homologous to bovine/caprine and human syntenic chromosomal segments (BTA Xp13/CHI Xq22 and HSA Xq27.3, respectively) may implicate parallel effects on fertility in these species [Danielak-Czech and Słota 2006, Słota *et al*. 2007a, b, Wittenberger *et al*. 2007, Usdin 2008, Kaczor *et al*. 2009, Danielak-Czech *et al*. 2010]. In view of CGG repeat expansion followed by Xq27.3 fragile site expression with concomitant ovarian insufficiency in humans, we can assume similar molecular background of sheep reproductive dysfunction in the form of repeating ineffective matings.

CONCLUSIONS

1. The results obtained suggest that chromosome X structural instability in sheep may be the cause of inappropriate expression of genes determining reproductive traits and increased rate of aneuploid germ cells related to prenatal and perinatal mortality.

2. The findings can be applied in further comparative cytomolecular studies in order to ascertain relationships between chromosome X instability and phenotypic effects in other farm animals.

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Streszczenie. Cytomolekularna analiza spontanicznych oraz indukowanych *in vitro* przedmutacyjnych zmian struktury heterosomu X u owcy romanowskiej ujawniła wysoki poziom niestabilności regionów Xq11, Xq22, Xq31 i Xq32/33 u niepłodnych maciorek. Najwyższą częstość defektów strukturalnych stwierdzono w regionie OAR Xq22 zawierającym niestabilne trzynukleotydowe powtórzenia CGG sekwencji regulatorowej genu *FRM1*, zidentyfikowane metodą *in situ* PCR. Uzyskane wyniki sugerują, że strukturalna niestabilność chromosomu X u owiec może być przyczyną nieprawidłowej ekspresji genów warunkujących cechy reprodukcyjne, jak również podwyższonego wskaźnika aneuploidalnych komórek rozrodczych i związanej z tym śmiertelności prenatalnej i okołoporodowej.

Słowa kluczowe: owce, niestabilność heterosomu X, łamliwy chromosom X, gen *FRM1*, tandemowe powtórzenia trzynukleotydowe