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CYTOMOLECULAR ANALYSIS OF CHROMOSOME X FRAGILITY IN SUBFERTILE SHEEP

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Chromosomal fragility is thought to be involved in etiology chromosome aberrations and karyotype evolution and fragile X underlay severe clinical pathology in mammals. The most studied fragile site, observed in cells exposed to mutagens/clastogens such as aphidicolin or folate-deficient conditions, is human FRAXA (co-expressed in Xq27.3 locus with trinicleotide CGG repeat expansion of FRM1 gene) associated with hereditary defects and reproductive dysfunction. In view of that clinical effects of such phenomenon in farm animals are not enough explained we performed cytomolecular analysis of spontaneous and in vitro induced chromosome X instability in sheep with reproductive problems. The studies in subfertile ewes resulted in pointing out chromosome Xq31, Xq33 and Xq22 regions as especially predisposed to structural defects. The latter, OAR Xq22 region comprising fragment of FRM1 gene, with fluorescence in situ labeled, trinucleotide CGG repeats was revealed to be the most unstable in infertile ewes. The results obtained suggest that fertility impairment in sheep may be a consequence of chromosome X fragility in regions comprising loci determining reproductive traits.

Key words: SHEEP, SUBFERTILITY, CHROMOSOMAL FRAGILE SITES, FRAGILE X, *FRM1* GENE, TRINUCLEOTIDE TANDEM REPEATS.

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. Thus, extensive studies have been undertaken on the fragile sites (FS) (nonrandom chromosomal breaks/gaps) in several *Bovidae* species regarding different methods of induction, and their clinical and biological significance (Riggs and Rønne, 2009). In result, bovine chromosome fragility (mainly chromosome X), induced by exposure to aphidicolin (APC — DNA polymerase inhibitor) or 5-fluorodeoxyuridine (FudR — folate synthesis antagonist) 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).

The aim of the present study was to evaluate spontaneous as well as APC- and folate-dependent chromosome X fragility in subfertile ewes, with special emphasis on OAR Xq22 region comprising fragment of *FRM1* gene, with fluorescence *in situ* labeled, trinucleotide CGG repeats.

Materials and methods

Animals: Cytogenetic evaluation was performed in population of 24 exterior normal, 2–5 years ewes of the old native Polish Heath sheep, taking into account individual reproductive

efficiency. The experimental group (n=12) included infertile ewes owing to ineffective matings (100 % in two years) and the control group (n=12) was composed of ewes having normal reproductive performance.

Chromosome preparation: Sheep metaphase chromosome slides 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). *IN situ PCR and detection*: The GCTCAGCTCGGTTTCGGTTTCACTTCCCGT (forward) and AGCCCCGCACTTCCACCACCAGCTCCTCCA (reverse) primers 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 thermal 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 hybridization signals were analyzed with fluorescence microscope equipped with the computer-assisted image analysis system LUCIA-FISH (Laboratory Imaging Ltd, Prague, Czech Republic).

Statistical analysis: 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 induced breaks/gaps in karyotype and separately in the pair of X chromosomes, calculated for each group and compared statistically between groups.

Table 1

Frequency of spontaneous and induced breaks/gaps in groups of ewes studied

Group	Spontaneous breaks/gaps		APC — sensitive breaks/gaps		Folate — sensitive breaks/gaps	
	Total % SD	X chromosome %	Total % SD	X chromosome %	Total % SD	X chromosome %
e	2,83±2,40	1,58	32,17*±11,09	13,21*	8,98±4,20	4,25*
c	1,50±1,56	1,08	22,01±8,0	8,15	6,02±2,99	2,58

Note: e — the experimental group; c — the control group; * — P≤0,05.

As shown in Table 1, the total frequencies of APC-sensitive breaks/gaps in the groups studied exceeded of folate–sensitive damages. Generally, fragile sites in X chromosomes occurred with frequency above 1 % within groups, but percentages of X-specific lesions were significantly higher (P≤0,05) in the experimental group comparing to the control one. The frequencies of breaks/gaps at excessively damaged Xq31, Xq22 and Xq33 regions, given in Table 2 and presented in Figure 1, differed on the statistically significant (P≤0,05) or highly significant (P<≤0,01) level between groups.

Table 2

Frequency of APC- and FudR-induced breaks/gaps at excessively damaged chromosome X regions in groups of ewes studied

Chromosome X region (description)	Breaks / gaps (%) — experimental group	Breaks / gaps (%) — control group
q31 (APC — sensitive)	5,33*±2,29	3,24±2,01
q22 (APC — sensitive)	9,01**±7,15	5,15±2,23
q33 (Folate — sensitive)	5,45*±2,38	3,12±1,96

Note: * — P≤0,05; ** — P<≤0,01

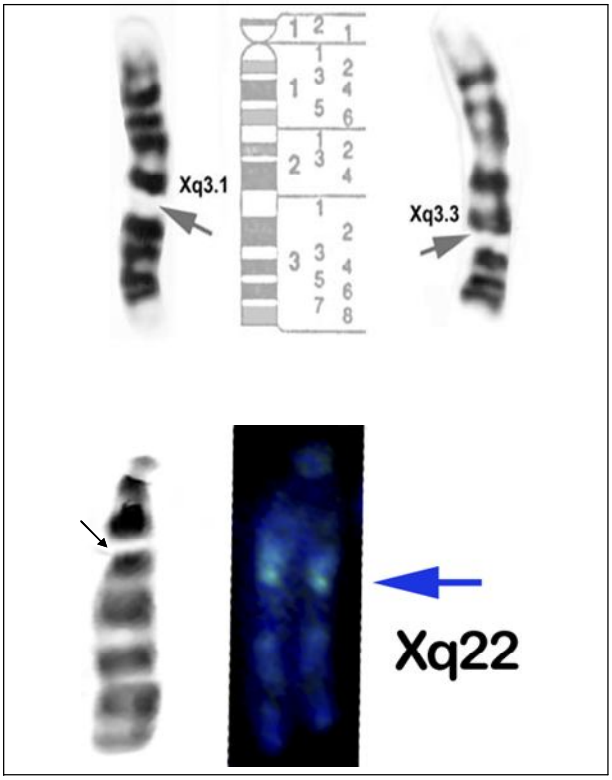

Fig 1. Highly expressive fragile sites in infertile ewes: OAR Xq31, Xq33 (upper) and OAR Xq22 (below), the latter comprising fragment of *FRM1* gene, with fluorescence *in situ* labeled, trinucleotide CGG repeats

The present studies showed APC- and folate-dependent X chromosome fragility in sheep, especially distinct in the group of ewes repeating ineffective matings. The Xq31and Xq33 bands have been proved to be the extremely expressive fragile sites in infertile ewes of the old native Polish Heath sheep. The results obtained confirmed our previous studies in this breed, supplemented by observations of 5-AZA/BrdU-sensitive fragility intensified distinctly in Xq32/33 regions of subfertile ewes (Słota and Danielak-Czech, 2002; Danielak-Czech and Słota, 2002, 2004). Interestingly, the sheep Xq31–33 chromosome segment (including mentioned above Xq32/33) is known to demonstrate synteny conservation and segment homology with the fragile cattle Xq26-31chromosome fragment, observable in some cases in the repeat breeding cows (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 *Bovidae* species, 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 *Bovids*, 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). In view of CGG repeat expansion followed by Xq27,3 fragile site expression concomitant ovarian insufficiency in humans, we can assume similar molecular background of sheep reproductive dysfunction in the form of repeating ineffective matings.

On the basis of results obtained it is possible to conclude that fertility problems in sheep may result from structural fragility of chromosome X regions with *loci* determining reproductive

traits. Nevertheless, these 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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ЦИТОМОЛЕКУЛЯРНИЙ АНАЛІЗ НЕСТІЙКОСТІ Х-ХРОМОСОМИ У ОВЕЦЬ СХРЕЩЕНИХ ПОРІД

Р е з ю м е

Нестійкість хромосоми пов'язують з відхиленням хромосоми та еволюцією каріотипу, також нестійка Х-хромосома лежить в основі складних клінічних патологій у ссавців. Найбільш нестійкою частиною клітини, вразливою до мутагенів/кластогенів таких як апідіколін та фолат-дефіцитні умови, це FRAXA у людини, що виражається у Xq27.3 розміщенні разом з триніклеотидом CGG повторним поширенням гену FRM1, це пов'язано з спадковими вадами та репродуктивною дисфункцією. З огляду на те, що цей клінічний ефект даного феномену у с.-г. тварин недостатньо вивчений ми представили цитомолекулярний аналіз спонтанної стимуляції хромосоми in vitro у овець з проблемами відтворення. У результаті досліджень на вівцях схрещених порід визначили, що ділянки хромосом Xq31, Xq33 та Xq22 особливо схильні до структурних порушень. У ділянці OAR Xq22, включаючи фрагмент гена FRM1, позначений in situ флуоресценцією, виявлено повтори тринуклеотиду CGG, який є дуже стабільним у неплідних овець. Одержані результати свідчать, що ослаблення плідності у овець може бути наслідком нестійкості Х-хромосоми у ділянках, які включають частинки, що визначають відтворювальну здатність.

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ЦИТОМОЛЕКУЛЯРНИЙ АНАЛИЗ НЕУСТОЙЧИВОСТИ Х-ХРОМОСОМЫ У ОВЕЦ СКРЕЩЕННЫХ ПОРОД

А н н о т а ц и я

Неустойчивость хромосомы связывают с отклонением хромосомы и эволюцией кариотипа, также неустойчивая Х-хромосома лежит в основе сложных клинических патологий у млекопитающих. Наиболее неустойчивой частью клетки, чувствительной к мутагенам/кластогенам таких как апидиколин и фолат-дефицитные условия, это FRAXA у человека, что выражается в Xq27.3 размещении вместе с тринуклеотидом CGG повторным распространением гена FRM1, это связано с наследственными недостатками и репродуктивной дисфункцией. Ввиду того, что этот клинический эффект данного феномена в с.-г. животных недостаточно изучен, мы представили цитомолекулярный анализ спонтанной стимуляции хромосомы in vitro у овец с проблемами размножения. В результате исследований на овцах скрещенных пород определили, что участки хромосом Xq31, Xq33 и Xq22 особенно склонны к структурным нарушениям. В участке OAR Xq22, включая фрагмент гена FRM1, обозначенный in situ флуоресценцией, выявлены повторы триниклеотида CGG, который является очень стабильным у бесплодных овец. Полученные результаты свидетельствуют, что ослабление плодотворности у овец может быть следствием неустойчивости Х-хромосомы в участках, которые включают частицы, что определяют воспроизводительную способность.

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