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[1]Departament of Animal Cytogenetics and Molecular Genetics,
National Institute of Animal Production, Krakowska 1, 32-083 Balice-Kraków,
e-mail: barbara.czech@izoo.krakow.pl
[2]Department of Pig Breeding and Production Technology,
University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin,
e-mail: marek.babicz@up.lublin.pl

BARBARA DANIELAK-CZECH[1], MAREK BABICZ[2],
BARBARA REJDUCH[1], ANNA KOZUBSKA-SOBOCIŃSKA[1]

Cytogenetic and molecular analysis of chromosome instability in cattle with reproductive problems

Cytogenetyczna i molekularna analiza niestabilności chromosomów u bydła z problemam w rozrodzie

Summary. The cytogenetic and molecular analysis of cattle chromosome instability revealed excessive fragility of chromosome X in q11, q24, q31 and p13 regions in cows with reproductive problems. The highest frequency of structural defects was found in BTAXp13 region containing *FRM1* gene *locus* with unstable trinucleotide CGG repeats which were mapped by *in situ* PCR method. The results obtained suggest that chromosome X structural instability may be a cause of defective expression of genes related with fertility and it leads to a decrease of reproductive efficiency. The present outcomes can be used in further studies on the cytogenetic and molecular substructure of chromosome instability phenomenon and their phenotypic effects in cattle.

Key words: cattle, fertility, chromosome instability, *FRM1* gene, trinucleotide tandem repeats

INTRODUCTION

The phenomenon of structural genome instability is proved to be involved in the etiology of chromosome fragility and genome rearrangement in mammals. Increased spontaneous chromosome fragility (mainly chromosome X) resulting from *in vivo* exposure to environmental mutagens or clastogens is considered to have phenotypic implications and impact on reproductive capabilities [Basrur and Stranzinger 2008]. Therefore, to determine cause-effect relationships of this phenomenon, extensive *in vitro* studies aimed to identify chromosomal *fragile sites* (*FS* – nonrandom chromosomal breaks/gaps) in livestock have been performed in the past few years. The investigations concerned the different modes of *FS* induction by *in vitro* exposure to chemical agents or specific cul-

ture conditions changing genome structure, based on DNA polymerase inhibition, nucleotide substitution or folate synthesis deficit [Riggs and Rønne 2009].

The phenomenon of structural chromosome instability in farm animals (including several *Bovidae* species) was previously reported to elicit frequent clinical pathologies or reproductive disturbances (especially chromosome X defects in subfertile cows) [Llambi and Postiglioni 1997, Słota *et al.* 2000; Danielak-Czech and Słota 2002, 2004]. The latest studies in domestic animals, like in humans, link regional chromosome X fragility with instability of tandem repeated sequences, e.g. trinucleotide CGG repeats expansion of the *FRM1* gene related with fragile X syndrome (leading to neurodegeneration and ovarian dysfunction) [Hagerman 2006, Wittenberger *et al*. 2007]. Homologous CGG repeats of this gene have been recently mapped to conserved bovine, ovine and caprine heterosome X regions (BTAXp13 and OAR/CHI Xq22, respectively) [Danielak-Czech and Słota 2006; Kaczor *et al*. 2009]. On the basis of subsequent investigations in sheep, this chromosome region (OARXq22) was defined as to be extremely susceptible to premutation structural changes associated with prevalent ewes' infertility [Danielak-Czech *et al*. 2010, 2011]. In cattle, clinical, reproductive and breeding consequences of structural chromosome instability still remain to be precisely determined.

The aim of the present paper was to perform cytogenetic and molecular analysis of spontaneous and *in vitro* induced chromosome instability in cattle and define potential influence of this phenomenon on fertility.

MATERIAL AND METHODS

Animals. Cytogenetic evaluation was performed in population of 28 exterior normal, 2–5 years old cows of Holstein-Friesian breed (divided into experimental and control groups taking into account individual reproductive efficiency). The experimental group (n = 14) involved the animals with reproductive problems expressed by high services/conception index, long calving interval and considerable losses of calves (%) resulting from abortions, stillbirths and early death after birth. The control group (n = 14) consisted of cows having normal reproductive performance.

Chromosome preparation. Cattle metaphase chromosome slides, both untreated and 24 h before harvesting exposed to 0.4 μM aphidicolin (APC - DNA polymerase inhibitor) or 0.1 μM 5-fluorodeoxyuridine (FudR - folate synthesis antagonist), were prepared following classical cytogenetic protocols of lymphocyte culture and banding techniques (GTG/ QFQ/DAPI). Karyotypes were arranged according to the international cattle karyotype standard.

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 of the homologous sequence [Troyer *et al.* 1994] 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 one-way analysis of variance - *F* test as well as χ^2 and Student's *t*-tests.

RESULTS AND DISCUSSION

No visible phenotype changes and stable karyotype rearrangements were found in the animal investigated. The average values of several reproductive parameters calculated for the groups of subfertile and normally fertile cows are presented in Table 1.

Table 1. Reproductive performance parameters in groups of cows studied
Tabela 1. Parametry użytkowości rozpłodowej w grupach badanych krów

Experimental group n = 14 Grupa doświadczalna n = 14	Control group n = 14 Grupa kontrolna n = 14
services / conception – 4.6 calving interval (in months) – 16.4 calves (stillborn/aborted/early dead) –20.0%	services / conception – 1.3 calving interval (in months) – 12.5 calves (stillborn/abotred/early dead) – 6.2%

Table 2. Frequency of spontaneous and induced *in vitro* chromosome structural defects in groups of cows studied
Tabela 2. Częstość spontanicznych oraz indukowanych *in vitro* strukturalnych defektów chromosomów w grupach badanych krów

Group n=14 Grupa n = 14	Spontaneous chromatid breaks/gaps Spontaniczne pęknięcia/ przewężenia chromatyd		APC-induced chromatid breaks/gaps Pęknięcia/przewężenia chromatyd indukowane ACP		Folate-sensitive chromatid breaks/gaps Folianowrażliwe pęknięcia/ przewężenia chromatyd	
	Total % SD	Heterosome X (%)	Total % SD	Heterosome X (%)	Total % SD	Heterosome X (%)
e.	5.49** ±3.36	2.53**	39.08* ±17.50	18.12**	14.09 ±6.22	6.55*
c.	3.15 ±2.73	1.12	27.23 ±15.04	11.50	9.95±6.30	3.46

e. – the experimental group / grupa eksperymentalna.
c. – the control group / grupa kontrolna.
*significant differences (P < 0.05) / różnice istotne (P < 0,05).
**highly significant differences (P < 0.01) / różnice wysoko istotne (P < 0,01).

The results of cytogenetic evaluation carried out in the experimental and control groups of cows are given in Table 2. The findings include the mean total frequency (%) of spontaneous and *in vitro* induced chromatid breaks/gaps identified in the whole karyotype and separately in the pair of X chromosomes, calculated for each group and compared statistically between groups. Generally, the frequencies of spontaneous and *in vitro* induced chromatid breaks/gaps were higher in the experimental group of cows than in the control one and the percentages of APC-sensitive breaks/gaps in the groups stud-

ied exceeded the proportions of folate–sensitive damages. As shown in Table 2, the frequencies of spontaneous and *in vitro* induced, both total and chromosome X-specific, structural defects (excluding the total number of folate-sensitive ones) differed statistically ($P \leq 0.05$ or $P \leq 0.01$) between the groups.

Table 3. Unstable chromosome X regions – frequency of *in vitro* induced structural defects in groups of cows studied
Tabela 3. Niestabilne regiony chromosomu X – częstość indukowanych *in vitro* defektów strukturalnych w grupach badanych krów

Chromosome X unstable regions Niestabilne regiony chromosomu X	Chromatid breaks/gaps (%) Pęknięcia/przewężenia chromatyd (%)	Chromatid breaks/gaps (%) Pęknięcia/przewężenia chromatyd (%)
	Experimental group n = 14 Grupa eksperymentalna n = 14	Control group n = 14 Grupa kontrolna n = 14
Xq11 (APC-induced)	4.33* ±1.80	2.24 ±1.02
Xq24 (folate-sensitive)	7.97** ±5.16	3.77 ±1.91
Xq31 (APC-induced)	5.40* ±2.27	3.09 ±1.74
Xp13 (folate-sensitive)	8.75** ±4.22	4.17 ±2.18

*significant differences ($P < 0.05$) / różnice istotne ($P < 0,05$).
**highly significant differences ($P < 0.01$) / różnice wysoko istotne ($P < 0,01$).

The most expressive *fragile sites*, shown in Figure 1, occurred with frequency above 1% within groups. The percentages of chromosome 1-, 2- and X-specific lesions were higher in subfertile cows. The frequencies of heterosome X-specific defects, given in Table 3, were significantly or highly significantly higher in the experimental group comparing with the control one. The highest frequency of structural defects was found in karyotype of cows with reproductive problems in BTAXq11, BTAXq24, BTAXq31 bands and especially BTAXp13 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 D).

The excessively damaged X-specific *fragile sites* found out in subfertile cows correspond to the spontaneous or previously *in vitro* classified structural instability associated with phenotypic abnormalities and fertility disturbances in numerous cattle breeds. Some cases of X chromosome fragility observable close to the centromere in a pale staining Q band (Xq11) and classified as folate-deficient FraX have been registered in Holstein cows with congenital malformations - baldy calf syndrome and polymelia [Uchida *et. al*. 1986, Nowacka *et al*. 2007]. Further studies certified the instability of the pericentromeric area (Xqcen), manifesting itself mainly in Black and White and Holstein-Friesian cows displaying frequent repeat breeding or recurrent stillbirths [Llambí and Postiglioni 1997, Danielak-Czech and Słota, 2002, 2004, Słota and Danielak-Czech 2002]. The other spontaneous fragile Xq regions located between positive and negative G bands – Xq23/q24 as well as in negative G-band Xq31 were identified in crossbred cows (Black and White × Holstein-Friesian, Black and White × Piedmontese) showing long calving interval and reduced rate of non-return for services after artificial insemination [Słota *et. al*. 2000]. The Xq31 hypersensitiveness associated with various forms of sub- or infertil-

ity has been reported earlier in Japanese Black, Holstein-Friesian and Uruguayan Creole cows [Uchida *et. al*. 1986, Llambí and Postiglioni 1997, Rincón *et.al*. 1997]. In the following experiments carried out *in vitro* in Black and White as well as Holstein-Friesian cattle the Xq23/q24 and Xq31 bands were ascertained as BrdU/5-AZA-sensitive and APC-dependent bovine *fragile sites* [Llambi *et. al*. 1999, Danielak-Czech and Słota 2002, 2004, Słota and Danielak-Czech 2002, Rodriguez *et al*. 2002). Moreover, unusual spontaneous instability of Xq24 region was revealed in Holstein-Friesian calf with rare congenital defect – amelia [Szczerbal *et al*. 2006]. Likewise, in our investigations involving population of Holstein-Friesian cattle, Xq24 *fragile site* together with the other excessively unstable regions: Xq11, Xq31 and Xp13 were identified in cows with notable reproductive problems: high services/conception index, long calving interval and considerable losses of calves.

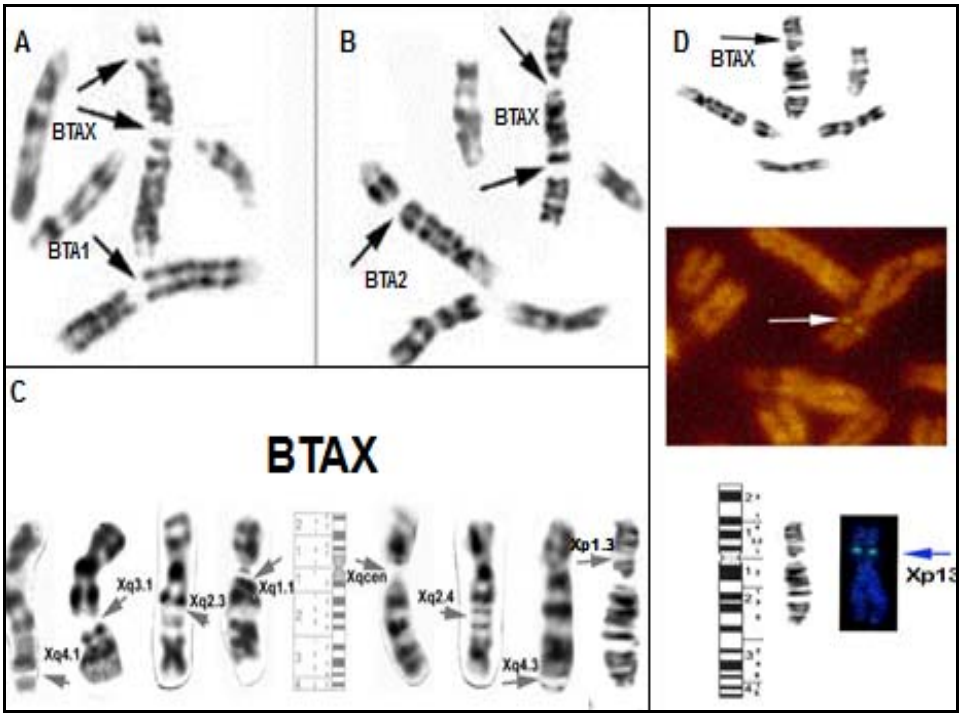

Figure 1. Fragile sites (*FS*) in karyotype of cows with reproductive problems: examples of *FS* on autosomes and heterosome X – BTA1, BTA2, BTAX (A, B); chromosome X-specific *FS* –APC-induced (on the left), folate-sensitive (on the right) (C); unstable BTAXp13 region comprising trinucleotide CGG repeats of *FRM1* gene regulatory sequence, mapped by *in situ* PCR method (D)
Rysunek 1. Miejsca łamliwe (*FS)* w kariotypie krów z problemami w rozrodzie: przykłady *FS* na autosomach i heterochromosomie X – BTA1, BTA2, BTAX (A, B); *FS* specyficzne dla chromosomu X – indukowane APC (po lewej), folianowrażliwe (po prawej) (C); niestabilny region BTAXp13 zawierający trzynukleotydowe powtórzenia CGG sekwencji regulatorowej genu *FRM1*, wymapowane metodą *in situ* PCR (D)

Both unstable bovine Xp13 and adequate ovine Xq22 region involving *FRM1 locus* (related with decreased fertility) represent evolutionary conserved *fragile site*, preserved in most *Bovidae* species [Danielak-Czech and Słota 2006, Kaczor *et al.* 2009, Danielak-Czech *et al*. 2010, 2011]. Homologous human *fragile site* (Xq27.3) comprising *FRM1* gene, due to expansion of highly polymorphic 5'UTR CGG repeats, affects many biological processes (chromosome fragility, gene silencing, transcription, translation or splicing modulation and cell architecture) resulting in genetic disorders and reproductive disturbances [Deelen *et al*. 1994, Hagerman 2006, Wittenberger *et al.* 2007, Usdin 2008]. The excessive instability of this orthologous bovine X chromosome region is supposed to implicate parallel effects on reproduction in *Bovidae* species. Thus, *FRM1 locus* may be a candidate gene for fertility impairment associated with chromosomal fragility in *Bovids*, just as *TRO* gene (trophinin) responsible for embryo implantation, localized in another unstable region – Xq25-33 conserved in genomes of *Bovidae* family [Asai *et al.* 2004]. Therefore, similar molecular background of bovine reproductive dysfunction in the form of repeating breeding and recurrent prenatal or perinatal mortality can be assumed. The findings can be applied in cytomolecular comparative studies to evidence relationships between chromosome instability and reproductive performance in the other breeding animals.

CONCLUSIONS

1. The results obtained suggest that chromosome X structural instability may lead to defective expression of genes related with fertility and decrease of reproductive efficiency.

2. The present outcomes can be used in further studies on cytogenetic and molecular substructure of chromosome instability phenomenon and their phenotypic effects in cattle.

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Streszczenie. Cytogenetyczna i molekularna analiza niestabilności chromosomów bydła ujawniła szczególną łamliwość chromosomu X w regionach q11, q24, q31 i p13 u krów z problemami w rozrodzie. Najwyższą częstość defektów strukturalnych indukowanych *in vitro* stwierdzono w regionie BTAXp13 zawierającym *locus* genu *FRM1* z niestabilnymi trzynukleotydowymi powtórzeniami CGG, które wymapowano metodą *in situ* PCR. Uzyskane wyniki sugerują, że strukturalna niestabilność chromosomu X u bydła może być powodem nieprawidłowej ekspresji genów związanych z płodnością i prowadzić do obniżenia wydajności reprodukcyjnej. Prezentowane wyniki mogą być wykorzystane w dalszych badaniach dotyczących cytogenetycznego i molekularnego podłoża zjawiska niestabilności chromosomów oraz jego fenotypowych efektów u bydła.

Słowa kluczowe: bydło, płodność, niestabilność chromosomów, gen *FRM1*, trzynukleotydowe powtórzenia tandemowe