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Embryos produced *in vitro* from bulls carrying 16;20 and 1;29 Robertsonian translocations: detection of translocations in embryos by fluorescence *in situ* hybridization

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Summary

Robertsonian translocation rob(16;20) in the heterozygous state was discovered in a subfertile bull of the Czech Siemmental breed. A chromosomal analysis of its family has shown that this dicentric fusion is formed *de novo*. The present experiments were designed to detect rob(16;20) and determine its incidence for *in vitro* produced embryos, using fluorescence *in situ* hybridization (FISH) and rob(1;29) as a detection control. To characterize semen of both bulls with the rob translocations, their sperm was examined for DNA integrity by the sperm chromatin structure assay (SCSA). For *in vitro* fertilization of oocytes, spermatozoa from a rob(16;20) bull carrier (Czech Siemmental breed) and those from a rob(1;29) bull carrier (Charolais breed) were used. Embryos at the 6- to 8-cell stage were cultured in a vinblastine-supplemented medium for 17 h, and embryos at the blastocyst stage were cultured in a colcemide-supplemented medium for 4 h. The embryos were fixed in methanol and acetic acid with Tween-20. Painting probes for chromosomes 16 (Spectrum Green) and 20 (Spectrum Orange) and chromosomes 1 (Spectrum Orange) and 29 (Spectrum Green) were simultaneously hybridized. In the embryos derived from the rob(16;20) bull, the presence of this translocation was not detected. On the other hand, 52.5% of the embryos derived from the rob(1;29) bull were translocation carriers. There was no significant difference in the frequency of this translocation between early and advanced embryos.

Keywords: Cattle, Detection, FISH, *In vitro* embryos, Robertsonian translocations

Introduction

Robertsonian translocations are the most common chromosomal abnormalities in cattle. They result from breakage and subsequent fusion of the centromeric regions of two acrocentric chromosomes. They are widespread, affecting different breeds of cattle (Popescu, 1996); more than 25 different types of centric fusion have been identified so far. The translocation is rapidly disseminated by artificial insemination due to the lack of its effect on the phenotype. Most of their carriers are translocation heterozygotes (De Luca *et al.*, 2002). However, some types of centric fusion are not so widespread and their role in reproduction has not been fully elucidated yet. For instance, it has been described that a slight reduction in fertility is associated with rob(1;29) and rob(7;21) (Hanada, 1998). These translocations are inherited by Mendelian inheritance and half the progeny are translocation heterozygotes (Hanada *et al.*, 1995; Hanada, 1998).

The rob(16;20) translocation was first described in the male offspring of a German Siemmental bull and a Czech Siemmental cow (Rubes *et al.*, 1996). A chromosomal analysis of the bull's father could not be performed; however, the analysis of 26 paternal half-brothers and the bull's mother showed that this was a *de novo* Robertsonian translocation and c-banding revealed its dicentric nature. Although its sperm showed normal concentration and motility, and only a slight increase in the percentage of pathological spermatozoa, the bull achieved poor results in insemination (36.4% non-return rate). Ten cows experimentally inseminated with this bull's sperm produced only four calves with a normal karyotype and therefore the bull was eliminated from breeding. However, it is possible to study the effect of this translocation on male fertilization

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ability by the method of *in vitro* oocyte fertilization. A potential relationship between this translocation and a low embryo development has already been described (Rubes *et al*., 1999) but the incidence of the translocation has not been verified.

The present experiments were designed to detect rob(16;20) and, by the method of fluorescence *in situ* hybridization (FISH), to determine its incidence in embryos produced *in vitro*; rob(1;29) served as a detection control. To assess the quality of semen of the rob(16;20) and rob(1;29) carriers, their sperm was characterized in terms of DNA integrity by the sperm chromatin structure assay (SCSA).

Materials and methods

Preparation and treatment of embryos

Cumulus–oocyte complexes (COCs) were obtained, matured and fertilized by the standard protocol described previously (Machatkova *et al*., 2000).

Spermatozoa for oocyte fertilization were from the rob(16;20) bull (Czech Siemmental breed) and the rob(1;29) bull (Charolais breed).

Eight-cell embryos and blastocysts (days 2 and 7 after fertilization, respectively) were cultured either in a medium with vinblastine (0.1 µg/ml) for 17 h, or in a medium with colcemide (0.05 µg/ml) for 4 h.

Chromosomal analysis of embryos

Fixation of embryos

Embryos were placed in a hypotonic solution (1% sodium citrate) for 10 min. Then they were prefixed in a mixture of acetic acid, methanol and water (1:4:5) for 1 min and subsequently fixed in methanol and acetic acid (3:1) containing Tween-20 (0.1%). They were washed and spread onto a slide, each slide then being fixed in methanol and acetic acid (3:1) for 4 h. To remove cytoplasm residue, the slides were treated with pepsin (0.2 mg/ml in 0.01 M HCl, at 37 °C for 15 min) and subsequently rinsed with phosphate-buffered saline (PBS) for 5 min. They were fixed in formaldehyde for 10 min, rinsed with PBS for 5 min and dehydrated gradually in an alcohol series (70%, 85% and 96%).

Fluorescence in situ hybridization (FISH)

Painting probes were prepared from laser-microdissected metaphase chromosomes by DOP-PCR (Kubickova *et al*., 2002).

Probes for chromosomes 16 (Spectrum Green) and 20 (Spectrum Orange) or for chromosomes 1 (Spectrum Orange) and 29 (Spectrum Green) were used in a double-colour FISH. Ten microlitre amounts of the hybridization mixture consisting of 50% formamide, 2× SSC, 10% dextran sulfate, 7 µg salmon sperm DNA, 10 µg calf thymus DNA and 0.6 µl probe were denatured, preannealed at 37 °C for 80 min and used for standard hybridization (Trask, 1991).

A BX60 Olympus microscope with fluorescence filters for fluorophores DAPI, FITC and Texas Red was used. Metaphases were recorded with a CCD camera and analysed by computer software (ISIS, MetaSystems, Altlussheim, Germany).

Analysis of sperm DNA integrity

Sperm chromatin structure assay (SCSA)

An increased susceptibility of altered DNA (strand breaks) in sperm nuclear chromatin to *in situ* denaturation was measured by flow cytometry after staining with acridine orange (AO). Sperm chromatin damage was quantified by red/red and green fluorescence. The semen was characterized by the percentage of mature cells with non-detectable DNA fragmentation, the percentage of mature spermatozoa with increased chromatin damage, with a detectable fragmentation index (DFI), and the percentage of immature sperm with high DNA stainability (HDS).

Semen treatment and sample measurement

Semen was rapidly thawed at 37 °C and then kept on ice. It was diluted with TNE buffer (0.015 M NaCl, 0.01 M Tris, and 0.001 M EDTA, pH 6.8) to bring sperm concentration to $1–2 \times 10^6$/ml. A 200 µl amount of sperm suspension was treated with 400 µl acid-detergent solution (0.08 M HCl, 0.1% Triton-X 100, pH 1.2) for exactly 30 s to induce DNA denaturation, and then 1.2 ml AO (6 µg/ml) was added.

Samples were placed in a flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA, USA) and exposed to 488 nm monochromatic laser light. After 2.5 min, 5000 spermatozoa per sample were evaluated. The data were saved in an offline computer and analysed using a SCSA-Soft software package. All measurements were carried out in duplicate.

Results

Chromosomal analysis of embryos by FISH

A total of 48 embryos derived from the rob(16;20) bull were examined; of these, 22 were 6- to 8-cell embryos and 26 were blastocysts. None of them was found to carry rob(16;20).

Of 53 examined embryos derived from the rob(1;29) bull, 34 were at 6- to 8-cell stages and 25 were blastocysts. More than half of these embryos showed the presence of rob(1;29), as seen in Table 1 and Fig. 1. No statistically significant differences in the frequency of this translocation were found between early and advanced embryos.

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Table 1 Frequency of embryos with rob(1;29)

Developmental stage	Embryos examined (n)	Embryos with karyotype 59,t (n)	Embryos with a normal karyotype (n)	Frequency of rob(1;29) (%)
6- to 8-cell embryo	34	18	16	52.9[a]
Blastocyst	25	13	12	52.0[a]
Total	59	31	28	52.5

[a] No statistically significant differences between values.

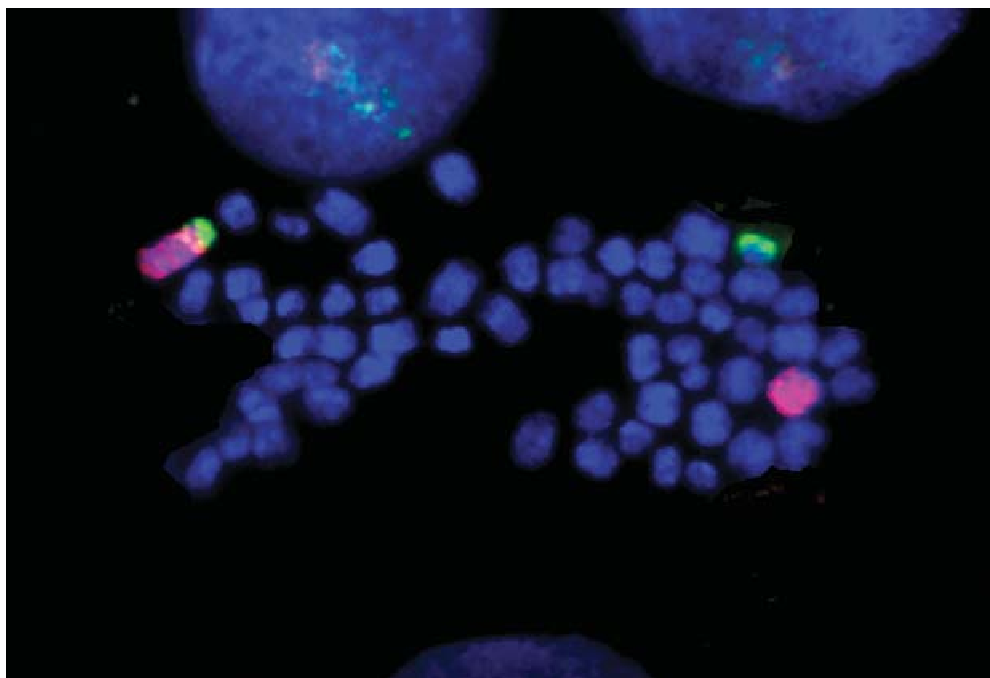

Figure 1 Mitosis in a blastocyst with a rob(1;29). Probes for chromosomes 1 (Spectrum Orange) and 29 (Spectrum Green) were used in double-colour FISH. Magnification, ×1000.

Characteristics shown by SCSA

In the semen of the rob(16;20) carrier, 5.3% of the spermatozoa showed a detectable fragmentation index (DFI) and 0.9% were evaluated as immature (HDS). These values were slightly higher in the sperm of the rob(1;29) carrier, i.e. 7.0% and 2.3% for DFI and HDS, respectively. The values characterizing the sperm of the rob(16;20) and rob(1;29) carriers were similar to those found in bulls of a normal population (5.7% and 1.4% for DFI and HDS, respectively).

Discussion

Studies on inheritance of Robertsonian translocations in cattle showed that in the offspring of bulls heterozygous for rob(1;29), rob(7;21) or rob(14;20), about 50% of the calves were translocation carriers, as theoretically predicted (McWhir *et al.*, 1987; Hanada, 1998). These results were confirmed by examination of *in vitro* produced embryos (Schmutz *et al.*, 1997).

However, no transmission of rob(16;20) was detected in our experiments with embryos produced by *in vitro* fertilization of oocytes with sperm from a rob(16;20) bull carrier. Since FISH with painting probes is a method facilitating examination of a high number of mitoses and an exact diagnosis of translocations, our negative finding cannot be attributed to an error in methodology. In addition, the high sensitivity of our method was confirmed by rob(1;29) transmission demonstrated, at an expected ratio, in embryos derived from the bull carrying this translocation; the numbers of embryos examined in the two experiments were comparable. Although not high, the number of embryos examined would have been sufficient to permit detection of rob(16;20) if this had been present.

Our results obtained *in vitro* are in agreement with previous findings that none of the calves born to mothers inseminated with sperm from the rob(16;20) bull carrier had this translocation. Because of the low non-return rate (36%), low efficiency of *in vitro* fertilization and low early embryo development it can be assumed that a certain, though very low, number

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of embryos free from this translocation may have developed after both *in vivo* and *in vitro* fertilization by sperm from the rob(16;20) bull (Rubes *et al*., 1996, 1999; Machatkova *et al*., 2004).

A reduction of fertility in translocation carriers can be due to the formation of chromosomally unbalanced gametes whose frequency varies widely depending on whether the carrier is the cow or bull (Schmutz *et al*., 1996; Hanada *et al*., 1995), and on the type of translocation (Hanada, 1998). The gametes with an unbalanced karyotype result in chromosomally abnormal embryos.

Logue & Harvey (1978) reported that the frequency of chromosomally unbalanced embryos was 6.4% when they were derived from rob(1;29) bull carriers, as compared with 2.8% of embryos derived from normal bulls; however, a bull heterozygous for rob(7;21) produced 7.9% of chromosomally unbalanced embryos (Hanada, 1994). Schmutz *et al*. (1997) described 22% of chromosomally unbalanced embryos derived from a bull with rob(14;20). In our rob(16;20) studies, about 40% of embryos showed an unbalanced karyotype, which implies a high proportion of chromosomally unbalanced spermatozoa involved in oocyte fertilization. Increased percentages of spermatozoa with abnormal chromatin were found in bulls with lesser fertility (Bochenek *et al*., 2001). An increased proportion of immature spermatozoa in semen may also reduce the efficiency of *in vitro* fertilization (Larson *et al*., 2000). To characterize semen quality from this point of view, we assessed sperm chromatin integrity by the SCSA in both the rob(16;20) and rob(1;29) bulls. In comparison with a population of fertile bulls (Rybar *et al*., 2004), neither of them showed a significantly higher number of spermatozoa with DNA damage or immature spermatozoa.

Transmission of Robertsonian translocations to embryos produced *in vitro* was studied by FISH with the use of probes prepared for chromosomes 16, 20, 1 and 29 in our laboratory. In the *de novo* rob(16;20) translocation, its transmission to developing embryos was not detected; in contrast, the transmission of rob(1;29) was confirmed according to a theoretical prediction.

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