Frequency of aneuploidy in in vitro matured MII oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds as determined by dual color fluorescent in situ hybridization (FISH)

D. Nicodemo, A. Pauciullo, G. Cosenza, V. Peretti, A. Perucatti, G.P. Di Meo, L. Ramunno, L. Iannuzzi, J. Rubes, D. Di Berardino. Frequency of aneuploidy in in vitro–matured MII oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds as determined by dual-color fluorescent in situ hybridization. Theriogenology, 73 vol.4, year 2010, 523–529
DOI: 10.1016/j.theriogenology.2009.10.007

Frequency of aneuploidy in in vitro–matured MII oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds as determined by dual-color fluorescent in situ hybridization

D. Nicodemo a, A. Pauciullo a, G. Cosenza a, V. Peretti b, A. Perucatti c, G.P. Di Meo c, L. Ramunno a, L. Iannuzzi c, J. Rubes d, D. Di Berardino a,*

a Department of Soil, Plant, Environment and Animal Production Sciences, University of Naples ‘‘Federico II,’’ Portici, Italy

b Department of Animal Science and Food Inspection, University of Naples ‘‘Federico II,’’ Naples, Italy

c Research National Council (CNR), ISPAAM, Laboratory of Animal Cytogenetics and Gene Mapping, Naples, Italy

d Veterinary Research Institute, Brno, Czech Republic

Abstract

The current study was undertaken to investigate the aneuploidy rates in in vitro–matured meiosis II (MII) oocytes and corresponding first polar bodies in two dairy cattle (Bos taurus) breeds by using dual-color fluorescent in situ hybridization (FISH). A total of 159 and 144 in vitro–matured MII oocytes of the Italian Friesian and Italian Brown breeds, respectively, were obtained according to the standard methods and analyzed by FISH using ‘‘Xcen’’ and ‘‘5’’ chromosome-specific painting probes, produced by chromosome microdissection and Degenerate Oligonucleotide Primer-Polymerase Chain Reaction (DOP-PCR). Oocytes with unreduced chromosome number were 10.1% and 16.7% in the two breeds, respectively. To avoid bias due to possible artifacts, the aneuploidy rates were determined by analyzing only oocytes with the corresponding polar bodies. In the Italian Friesian, 100 of 143 (69.9%) secondary MII oocytes showed clear MII plates with corresponding first polar bodies and were scored for aneuploidy detection; one oocyte was ‘‘nullisomic’’ for chromosome X (1.0%) and one ‘‘disomic’’ for chromosome 5 (1.0%). In the Italian Brown, 100 of 120 (83.3%) MII oocytes with corresponding first polar bodies were analyzed; one oocyte was nullisomic (1.0%) and one was disomic (1.0%), both for chromosome 5. Totally, 303 oocytes were analyzed, 40 of which showed an unreduced chromosome complement (13.2%); of 200 MII oocytes with the corresponding first polar bodies, the aneuploidy rate (nullisomy + disomy) for the two chromosomes scored was 2%. Assuming that each chromosome is equally involved in aneuploidy, it results that in cattle oocytes matured in vitro, at least 30% of the oocytes (1 x 30 haploid chromosomes) should be aneuploid. Premature separation of sister chromatids (PSSC) was also observed in 2% of the oocytes in the Italian Friesian breed involving chromosome 5 and in 1% of the Italian Brown breed involving the X chromosome. Estimation of the ‘‘baseline’’ level of aneuploidy in the in vitro–matured oocytes of the various domestic animal species and breeds is, to our opinion, a useful reference for improving the in vitro production of embryos as well as for monitoring future trends of the reproductive health of the species/breeds engaged in zootechnical productions, especially in relation to management errors and environmental hazards.

Keywords: Aneuploidy; Bovine oocytes; Cattle breeds; Diploidy; FISH analysis; Polar bodies

1. Introduction

Bovine (Bos taurus) oocytes have mainly been investigated in the past for studying meiotic progression and maturation under in vitro conditions [1–7]. Most of the authors analyzed only the ploidy (reduced/unreduced) status of the oocytes, whereas only two articles [8,9] reported on the aneuploidy rates. All these works used the conventional air-drying method [10], which, as is known, allows visualization and counting of metaphase chromosomes at the MII stage. This method, however, has serious limitations, due partly to the quality of the spreading, which is not always optimal (overlapping chromosomes, presence of cytoplasmic residual, compacted metaphases) and partly to the fact that it induces chromosomal loss due to spreading.

Under these circumstances, the aneuploidy rate can be underestimated or overestimated. In addition, the chromosomal material of the corresponding first polar body cannot be analyzed because usually the chromosomes are too condensed and overlapped. The possibility to detect aneuploidy also in interphase cells (i.e., without the need to display metaphase chromosomes) came along with the fluorescence in situ hybridization (FISH) technique [11] by using chromosome-specific ‘‘painting’’ probes or Bacterial Artificial Chromosomes (BACs). This technique, if applied to MII oocytes with the corresponding first polar body, can provide a more precise estimation of aneuploidy, because the lack of any chromosome in the MII metaphase (nullisomy) should have its counterpart in the corresponding polar body, which should therefore result disomic, and vice versa. The limiting factor of this technique, however, is the scarce availability of the chromosome-specific probes. The PRINS technique [12] is another interesting way to analyze aneuploidy, but because there are no specific reports on domestic animals, we preferred to use the FISH approach. Because all the information we have to date on the aneuploidy rates in cattle oocytes has been collected by using the conventional air-drying method, we decided to review the available data in cattle aneuploidy by using the more resolutive FISH technique applied on in in vitro–matured MII oocytes plus first polar bodies by using

painting probes from chromosome X and chromosome 5 prepared by chromosome microdissection and DOP-PCR. These probes were chosen because they both provided strong and specific signals. Furthermore, while the Xcen probe was previously used for sperm FISH analysis [13], the probe for chromosome 5 was specifically prepared for this purpose.

The current contribution analyzes the rate of aneuploidy in the Italian Friesian and Italian Brown cattle breeds to estimate the ‘‘baseline’’ level of aneuploidy in the in vitro–matured oocytes of the species Bos taurus. These breeds have been chosen because of their worldwide importance for milk production. Once defined, this baseline level could be used as a reference for improving the in vitro production of embryos as well as for monitoring future trends of the reproductive health of the species/breeds engaged in zootechnical productions, especially in relation to management errors and environmental hazards.

2. Materials and methods

2.1. Karyotyping of donor cows

Cows ready for slaughtering were previously karyotyped according to standard methods [14]. The donor cows used in this study were all karyologically normal.

2.2. Collection of ovaries and oocytes and in vitro maturation

Ovaries were collected from slaughtered cows of the Italian Friesian and Italian Brown breeds, aging between 12 and 36 mo and transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) were collected through aspiration with 21-gauge needles, washed in TC-199 medium (No. M2154; Sigma, St. Louis, MO, USA), and examined on Petri dishes under a stereomicroscope. Only oocytes with compact-intact cumulus cell layers and good morphology were selected for the study. Groups of oocytes selected from each donor were transferred into 50-mL droplets of maturation medium consisting of TC-199 medium + 10% fetal bovine serum (No. 10106-151; Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 0.5 mg/mL follicle-stimulating hormone (FSH; No. F8174;

Sigma), 5 mg/mL luteinizing hormone (LH; No. L5269; Sigma), and 1 mg/mL estradiol (No. E2257; Sigma), covered with sterile mineral oil (No. M5310; Sigma) and allocated in a humidified atmosphere containing 5% CO2 in air at 39 8C for 24 h.

2.3. Oocyte fixation

After 24 h maturation, the COCs were incubated for a few minutes in a hyaluronidase solution (1 mg/mL; No. H4272; Sigma) to remove the cumulus cells, washed in Phosphate Buffered Saline (PBS), and exposed to a hypotonic sodium citrate solution (0.8% wt/vol) for 3 min, followed by KCl (75 mM) treatment for 3 min. The fixation was carried out using cold methanol/glacial acetic acid (3:1) solution. Oocytes were individually fixed at the center of a precleaned slide, air-dried, and kept at -20°C until analysis.

2.4. Chromosome microdissection and probes preparations

Metaphase cells for the production of probes via microdissection were prepared according to the standard cytogenetic techniques [14]. For microdissection, the fixed lymphocyte suspension was spread onto a precleaned 24 x 60 mm coverslip, which was then airdried and treated for GTG-banding. The Xcen probe was produced by isolating the pericentromeric region, corresponding with the centromere and with the region Xp11-14 of the standardized GTG-banded karyotype [13]; the probe for chromosome 5 was produced by scraping the entire chromosome. Microdissected chromosomes were amplified following the protocol of Engelen et al. [15]. Probes were labeled with dUTP-11-digoxigenin (chr. Xcen) and dUTP-16-biotin (chr. 5) (No. 11558706910 and No. 11093070910, respectively; Roche, Mannheim, Germany) in a second DOP-PCR reaction using 2 mL of products from the first reaction as template.

2.5. In situ hybridization

The Xcen and 5 probes were hybridized simultaneously on metaphase plates for validation and subsequently used for oocytes analysis. Probes were precipitated in the presence of 10 mg salmon sperm DNA (No. D7656; Sigma) and 10 mg of calf thymus DNA (No. D8661; Sigma) dissolved in 15 mL hybridization solution (50%formamide in 2X SSC + 10% dextran sulfate; No. F7503 and No. D8906, respectively; Sigma) (SSC – Standard Saline Citrate), denatured at 72 8C for 10 min, and incubated at 37 8C for 90 min. Fixed oocytes were denatured for 2 min in a solution of 70% formamide in 2X SSC (pH 7.0) at 72 8C. The hybridization mixture containing the Xcen and 5 probes was applied on the slides and covered with 24 _ 24 mm coverslips. The slides were hybridized in a moist chamber at 37 8C overnight. After hybridization and slide washing, the biotin-labeled probe was revealed using a green Alexa 488 fluorochrome conjugated to streptavidin (No. S-11223; Invitrogen, Carlsbad, CA, USA), and the digoxigenin-labeled probe was revealed using a red rhodamine fluorochrome conjugated to an antidigoxigenin antibody from sheep (No. 11207750910; Roche). Slides were counterstained with DAPI (40,60-diamidino-2-phenylindole, 0.24 mg/mL) (No. D9542; Sigma) in Antifade (No. H1000; Vector Laboratories, Burlingame, CA, USA).

2.6. Fluorescence analysis and scoring

The slides were observed at _ 100 magnification with a Leica (Wetzlar, Germany) DMRA fluorescence microscope equipped with DAPI, Fluorescein isothiocyanate (FITC), and Texas Red (TXRD) specific filters, the DAPI/FITC/TXRD triple filter, and phase-contrast optics. Digital images were captured using the Leica Q4000 software. To avoid possible bias, reduced secondary oocytes without the corresponding first polar bodies were excluded from the analysis. An oocyte was defined as ‘‘nullisomic’’ when one of the two signals (either X or 5) was lacking from the MII plate but present twice in the corresponding polar body; vice versa, an oocyte was defined as ‘‘disomic’’ when one extra signal (either X or 5) was present in the MII plate but absent from the polar body. Chi-square analysis was used for statistical analysis of data.

3. Results and discussion

The Xcen and 5 probes were hybridized simultaneously on cattle metaphase preparations for validation of the probes; Fig. 1 shows the Xcen and 5 painting probes, the DAPI staining, and their diagrammatic representation. Fig. 2 shows normal and abnormal in vitro–maturedMII oocytes after FISHby using Xcen and 5 chromosome-specific painting probes. The detailed results are shown in Table 1. Animals with less than four reduced secondary oocytes with analyzable polar bodies (PBs) were grouped. Totally, 549 COCs were collected through aspiration (295 and 254 in the Italian Friesian and Italian Brown, respectively). On the average, 13 COCs were recovered fromeach subject (range, 6 to 34). The percentage of COCs selected for in vitro maturation was around 70% in both breeds, but it was different among the subjects (range of selected COCs varied between 54% and 82%). Of 204 and 179 COCs selected for maturation, respectively, in the Italian Friesian and Italian Brown, 159 and 144 reached the MII stage; the efficiency of the in vitromaturation process was thus78% and 80% in the two breeds, respectively. Individually, the maturation rate varied between 57% and 100% among the subjects. Significant interindividual differences were found in the yield of in vitro–matured MII oocytes in the Italian Friesian as well as in the Italian Brown breed. As shown, these differences are mainly due to the number of oocytes obtained fromindividual donors and selected for in vitro maturation and partly due to the different efficiency of the in vitro maturation process in the different subjects. In the Italian Friesian, 16 oocytes of 159 showed an unreduced chromosome number (10.1%); of 100 MII + PB analyzed, one oocyte was nullisomic for the X chromosome and one disomic for chromosome 5, with an overall frequency of aneuploidy (nullisomy + disomy) of 2%; other two oocytes (2%) were affected by PSSC involving chromosome 5. In the Italian Brown, 24 oocytes of 144 showed an unreduced chromosome number (16.7%); of 100 MII + PB analyzed, one oocyte was nullisomic for chromosome 5, and one was disomic for the same chromosome, with an overall frequency of aneuploidy of 2%; only one oocyte (1%) showed PSSC involving the X chromosome.

By considering the two breeds together, on the average, 40 oocytes of 303 (13.2%) were found to be unreduced; of 200 MII + PB analyzed, 2 oocytes were nullisomic (1%), 2 oocytes were disomic (1%), with an overall aneuploidy rate, for these two chromosomes, of 2%, nullisomy and disomy being equivalent (i.e., 1% each). Totally, chromosome 5 showed a higher frequency of aneuploidy compared with that of the X chromosome (1.5% vs. 0.5%, respectively), but this difference was not statistically significant. The results of the current study demonstrated that in bovine oocytes matured in vitro, the mean rate of unreduction was on the average 13.2%. This value is ‘‘within’’ the interval already reported in the literature (around 8% to 12%) by use of conventional methods [3,5–8]. Considering the animal individually, 54% of the subjects showed one or more unreduced oocytes. Again, this value is within the range of other studies [4,7]. In the current contribution, the Brown breed showed a higher frequency compared with the Friesian (16.7% vs. 10.1%), but the difference was not statistically significant.

Interestingly, chromosome 5 was found to be three times more involved in aneuploidy compared with the X chromosome (1.5% vs. 0.5%, respectively); even though this difference was not statistically significant, it is indicative of the existence of interchromosomal differences in the rate of nondisjunction. Whereas in humans the interchromosomal differences have already been demonstrated [16,17], in domestic animals to date they have only been suggested.

By assuming an average rate of aneuploidy of 1% per chromosome and an equal probability of each chromosome being involved in aneuploidy, it results that in cattle, nearly 30% (1 x 30 haploid chromosomes) of the in vitro–matured oocytes might be aneuploid.

This estimate is higher compared with 5.8%and 7.1% previously reported in cattle by Yadav et al. [8] and by Lechniak and Switonski [9], respectively, by using conventional cytogenetic methods but very close to that obtained in the pig by using the FISH technique. In fact, Vozdova´ et al. [18] estimated that about 27% of the in vitro–matured pig oocytes are aneuploid, whereas conventional methods indicated an average frequency of 4% to 5% [19,20]. In humans, the aneuploidy rates vary among different laboratories, with the highest value reaching 47% [17]. By considering the results of this

study in cattle and those reported by Vozdova et al. in the pig [18], it seems that the FISH method overestimates -compared with the classic method- the incidence of aneuploidy, but this is simply due to the extrapolation from only two or three chromosomes, under the general assumption of no interchromosomal differences (i.e., equal likelihood for each haploid chromosome). This assumption, however, may not be true, and future studies should focus on this topic by using FISH with probes for all the chromosomes. Balanced PSSC was also observed in 2% of the oocytes in the Italian Friesian breed involving chromosome 5 and in 1% of the Italian Brown breed involving the X chromosome. A balanced PSSC is characterized by balanced chromosomal sets with separation of sister chromatids. This anomaly is not considered responsible for aneuploidies, although it may indicate a predisposition to nondisjunction. On the contrary, unbalanced PSSC can lead to embryonic aneuploidy in 50% of the cases, depending upon the behavior of the extra chromatid during the second meiotic division. No oocytes with unbalanced PSSC were observed in this study.

As known, aneuploidy in in vitro–matured oocytes is strongly dependent upon the culture system [21]; furthermore, it has been demonstrated that the percentage of embryos with chromosomally abnormal cells increases from 25% to 72% in in vivo–produced and in vitro–produced bovine embryos, respectively [22]. The whole in vitro embryo production system in cattle, starting from immature oocytes, has an efficiency of 30% to 40% [23]; this low efficiency is mainly due to the high rate of aneuploidy in the in vitro–matured oocytes used for fertilization. Further studies should, therefore, concern the optimization of the current in vitro culture system for oocyte maturation. Fertility in mammals is strongly affected by chromosomal abnormalities, which represent one of the major causes of embryonic mortality, as it has been demonstrated in humans [24–27] and in domestic animals [28–30]. Meiotic errors may originate spontaneously or may be due to the genotoxic effects of a variety of environmental mutagens, hormonal unbalancements, nutritional and dietetical mistakes, and so forth [17,31]. In any case, estimation of the baseline level of aneuploidy in the oocytes of the various domestic species and breeds is an essential step for improving the in vitro production of embryos destined to the embryo transfer industry as well as for monitoring future trends

of the reproductive health of domestic animals in relation to management errors and/or environmental hazards.

Acknowledgment

This work was supported by the financial contribution of the Ministry of Agricultural and Forestry Politics (MiPAAF) of Rome (SpermovoFISH project no. 291/7303/06), which is gratefully acknowledged.

References

[1] King WA, Xu KP, Sirard MA, Greve T, Leclerc P, Lambert RD, Jacques P. Cytogenetic study of parthenogenetically activated bovine oocytes matured in vivo and in vitro. Gamete Res 1988;20:265–74.

[2] Suss U, Wuthrich K, Stranzinger G. Chromosome configuration and time sequence of the first meiotic division in bovine oocytes matured in vitro. Biol Reprod 1988;38:871–80.

[3] Ectors FJ, Konlischer L, Jamar M, Herens C, Verloes A, Remy B, Beckers JF. Cytogenetic study of bovine oocytes matured in vitro. Theriogenology 1995;44:445–50.

[4] Sosnowski J, Switonski M, Lechniak D, Molinski K. Cytogenetic evaluation of in vitro matured bovine oocytes collected from ovaries of individual donors. Theriogenology 1996;45:865–72.

[5] Lechniak D, Switonski M, Sosnowski J. The incidence of bovine diploiid oocytes matured in vitro. Theriogenology 1996;46:267– 77.

[6] Ocana-Quero JM, Pinedo-Merlin M, Moreno-Millan M. Influence of follicle size, medium, temperature and time on the incidence of diploid bovine oocytes matured in vitro. Theriogenology 1999;51:867–72.

[7] Lechniak D, Kaczmarek D, Stanislavski D, Adamowicz T. The ploidy of in vitro matured bovine oocytes is related to the diameter. Theriogenology 2002;57:1303–8.

[8] Yadav B, King W, Xu K, Pollard J, Plante L. Chromosome analysis of bovine oocytes cultured in vitro. Genet Sel Evol 1991;23:191–6.

[9] Lechniak D, Switonski M. Aneuploidy in bovine oocytes matured in vitro. Chromosome Res 1998;6:504–1504.

[10] Tarkowski AK. An air-drying method for chromosome preparations from mouse eggs. Cytogenetics 1966;5:394–400.

[11] Langer PR, Waldrop AA, Ward DC. Enzymatic synthesis of biotin-labelled polynucleotides: novel nucleic acid affinity probes. Proc Natl Acad Sci U S A 1981;78:6633–7.

[12] Pellestor F, Girardet A, Andre´o B, Charlieu JP. A polymorphic alpha satellite sequence specific for human chromosome 13 detected by oligonucleotide primed in situ labelling (PRINS). Hum Genet 1994;94:346–8.

[13] Nicodemo D, Pauciullo A, Castello A, Roldan E, Gomendio M, Cosenza G, et al. X-Y sperm aneuploidy in two cattle (Bos taurus) breeds as determined by dual colour fluorescent in situ hybridization (FISH). Cytogenet Genome Res 2009;126: doi:10.1159/000245923, in press.

[14] Iannuzzi L, Di Berardino D. Tools of the trade: diagnostic and research applied to domestic animal cytogenetics. J Appl Genet 2008;49:357–66.

[15] Engelen JJ, Albrechts JC, Hamers GJ, Geraedts JP. A simple and efficient method for microdissection and microFISH. J Med Genet 1998;35:265–8.

[16] Kuliev A, Cieslak J, Verlinskly Y. Frequency and distribution of chromosome abnormalities in human oocytes. Cytogenet Genome Res 2005;111:193–8.

[17] Pacchierotti F, Adler I, Eichenlaub-ritter U, Mailhes JB. Gender effects on the incidence of aneuploidy in mammalian germ cells. Environ Res 2007;104:46–69.

[18] Vozdova´ M, Machatkova´ M, Kubikova´ S, Zudova´ D, Jokesova´ E, Rubes J. Frequency of aneuploidy in pig oocytes matured in vitro and of the corresponding first polar bodies detected by fluorescent in situ hybridization. Theriogenology 2001;56: 771–6.

[19] Mcgaughey RW, Polge C. Cytogenetic analysis of pig oocytes matured in vitro. J Exp Zool 1971;176:383–95.

[20] Sosnowski J, Waroczyk M, Switonski M. Chromosome abnormalities in secondary pig oocytes matured in vitro. Theriogenology 2003;60:571–81.

[21] A' Arabi SY, Roussel JD, Chandler JE. Chromosomal analysis of mammalian oocytes matured in vitro with various culture systems. Theriogenology 1997;48:1173–83.

[22] King WA. Chromosome variation in the embryos of domestic animals. Cytogenet Genome Res 2008;120:81–90.

[23] Lonergan P. State-of-the-art embryo technologies in cattle. Soc Reprod Fertil Suppl 2007;64:315–25.

[24] Hecht F, Hecht BK. Environmental chromosome damage. Am J Med Genet 1987;27:399–400.

[25] Martin HR, Ko E, Rademaker A. Distribution of aneuploidy in human gametes: comparison between human sperm and oocytes. Am J Med Genet 1991;39:321–31.

[26] Pellestor F. The cytogenetic analysis of human zygotes and preimplantation embryos. Hum Reprod Update 1995;1:581–5.

[27] Hassold TJ. Nondisjunction in the human male. In: Handel MA, editor. Meiosis and Gametogenesis. Academic Press; 1998. p.383–406.

[28] King WA. Chromosome abnormalities and pregnancy failure in domestic animals. Adv Vet Sci Comp Med 1990;23: 229–50.

[29] Kawarski SJ, Basrur PK, Stubbings RB, Hansen PJ, King WA. Chromosomal abnormalities in bovine embryos and their influence on development. Biol Reprod 1996;54:53–9.

[30] Vanroose G, de Kruif A, Van Soom A. Embryonic mortality and embryo-pathogen interactions. Anim Reprod Sci 2000;60–61: 131–43.

[31] Maihes JB, Marchetti F. Mechanisms and chemical induction of aneuploidy in rodent germ cells. Cytogenet Genome Res 2005; 111:384–91.