Lower quality bovine embryos may be successfully used for sex determination

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ABSTRACT: The aim of this study was to evaluate the effect of sex determination procedures in Day 7 to Day 8 bovine embryos of various quality. For the purposes of comparison we used high quality (HQ) as well as lower quality (LQ) embryos obtained from superovulated donors. The healthy embryonic cells isolated from HQ embryos and blastomeres protruding into the perivitelline space of LQ embryos, were analysed by polymerase chain reactions (PCR) using primers specific for the Y chromosome determinant. After microsurgical intervention and completion of sex determination, the female embryos were then transferred (ET) to synchronized recipients. A total of 310 embryos of HQ were used and gender was safely determined in 275 cases (88.7%). PCR analysis of extruded cells isolated from 170 LQ embryos was carried out with certainty only in 111 embryos (65.3%, $P < 0.01$). After ET of 122 HQ sex defined embryos, pregnancy was established in 69 recipients (56.6%). A similar conception rate 51.9% (27/52) was found after the ET of sex defined embryos designated as LQ. The accuracy of analysis was confirmed after calving and revealed that designated female sex coincided with 95.5% and 96.2% of calves when HQ and LQ embryos were transferred, respectively. Our results clearly show that a microsurgical technique in combination with PCR method represents a rapid and reliable approach for sex determination in HQ as well as in LQ preimplantation bovine embryos and can be used in field conditions for the regulation of the sex of progeny in selected herds.

Keywords: cattle; embryo; superovulation; sex determination; polymerase chain reaction; embryo transfer

Embryo transfer technology represents a powerful tool for the acceleration of various breeding programs in cattle. The exploitation of techniques allowing the sex determination of preimplantation embryos opens the way to the improvement of the genetic potential of cattle herds in shorter time intervals.

One of the first serious attempts to realize this intention was made by Edwards and Gardner (1967) who separated rabbit blastocysts according to the presence of Barr bodies, which represent the result of massive chromatin condensation of the X chromosome. In most other species, including farm animals, this method cannot be applied so simply due to the dense, non transparent cytoplasm. Likewise, cytogenetic methods based on the morphology of X and Y chromosomes (Singh and Hare, 1980; Rall and Leibo, 1987), did not live up to expectations. The same holds true for efforts directed at the immunological determination of specific sex antigen (White et al., 1982; Booman et al., 1989) and the detection of metabolic differences between male and female embryos (Williams, 1986). All the above mentioned methods, including the preparation of specific fragments for DNA hybridisation (Leonhard et al., 1987; Bondioli et al., 1989; Kobayashi et al., 1998), were time consuming and the variability of obtained results was so high as to make it unsuit-

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able for application in field conditions. Advances in DNA technology, however, opened up new possibilities for sex determination in mammals. Soon after the sequencing of the Y chromosome, specific DNA probes were used in PCR, and the first, highly promising results were published (Herr et al., 1990; Schroder et al., 1990). Recently, quite a few reports have indicated that under certain circumstances the sex of embryos may be determined with high accuracy and in acceptable time intervals (Thibier and Nibart, 1995; Lopes et al., 2001; Ekici et al., 2006; Yu et al., 2006).

Embryos flushed from superovulated donors are nearly exclusively used for the determination of sex in field praxis. It is generally known and our long term experience fully confirms (Lopatarova et al., 2006), that nearly all flushings contain embryos of very different quality. As a rule, sex determinations include embryos of high quality (HQ), which may be often in the minority. One of the aims of this investigation was to assess if also those of lower morphological quality (LQ) are potential candidates for microsurgery and subsequent treatments.

MATERIAL AND METHODS

Animals and treatment

Selected donors (n = 60) were stimulated according to the protocol described by Holy et al. (1990). Briefly, Holstein-Friesian cows were superovulated between days 8–12 of the oestrous cycle with eight doses of 480–560 IU (25–30 mg) per animal of FSH (Folicotropin, Spofa, Czech Republic) administered at 12-h intervals. Oestrus was induced by double prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) treatment (cloprostenol, Oestrophan, Spofa, Czech Republic) together with the fifth and sixth FSH injections. Three artificial inseminations were performed at 48, 60 and 72 h after the first application of $PGF_{2\alpha}$.

Embryo collection and scoring

Embryos were flushed with PBS (Dulbecco's Phosphate Buffered Saline, Live Technologies, Ltd., U.K.) + 1% FCS (foetal calf serum, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic) on Day 7 or Day 8 after the first insemination (Day 0). Flushing, as well as embryo isolation, was carried out as described by Holy et al. (1990).

Obtained embryos were washed with PBS + 10% FCS and classified according to their developmental stage and quality with respect to internationally accepted criteria. The designation high quality (HQ) embryo (n = 310) was assigned to morphologically intact compacted morulae, early blastocysts and expanded blastocysts (excellent or good, grade 1), whereas lower quality (LQ) embryos (n = 170) included those with few excluded blastomeres in the perivitelline space or blastocell cavity (fair, grade 2). Only these two groups were selected for microsurgical procedures.

Embryo biopsy

Microsurgical intervention was carried out with the twinner system (AB Technology, Inc., Pullman,

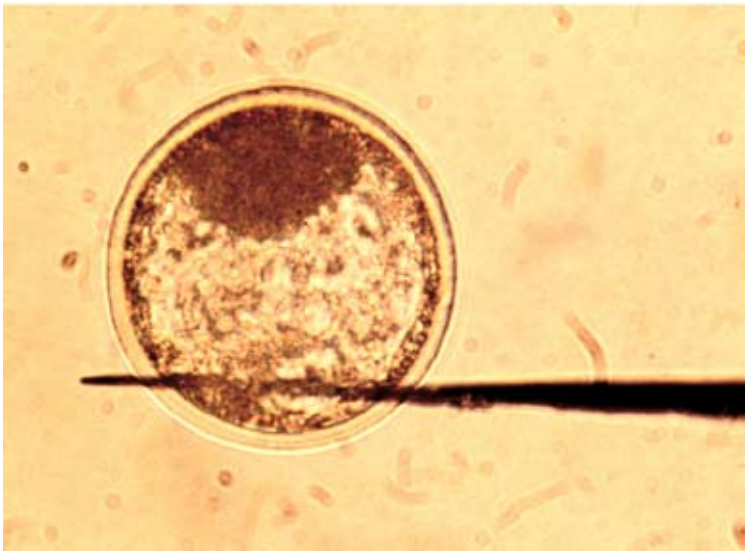

Figure 1. Biopsy of trophoblast cells from a high quality expanded blastocyst

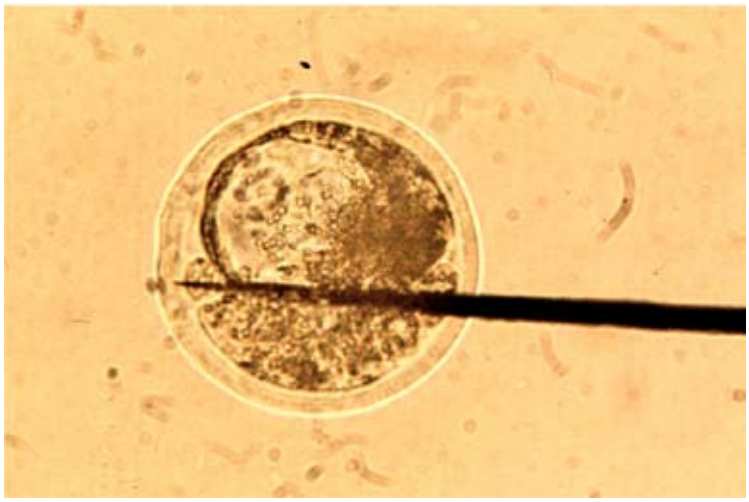

Figure 2. Evacuation of cells extruding the perivitelline space for sex determination in a lower quality blastocyst

WA). From D7 embryos (the compacted morulae and early blastocysts), a few surface embryonic cells (> 5), were removed by transzonal incision using a microsurgical blade. From D8 embryos (blastocysts), a few trophoblast cells were isolated (Figure 1). In LQ embryos, all cells extruding the perivitelline space were cut off and aspirated (Figure 2). Splitting Plus medium (AB Technology, Inc., Pullman, WA) or PBS without proteins was used for the immobilization of embryos. The isolated embryonic cells were analyzed immediately. During the analysis, the treated embryos were cultured in 4-well dishes containing 1 ml of PBS with 10% of FCS per well at room temperature (20–25°C).

Sex determination

The harvested cells were immediately transferred to microtubes containing Cresol red (o-Cresolsulfonephthalein). Embryo sexing was performed with a commercial polymerase chain reaction (PCR) kit using primers specific for the Y chromosome determinant (YCD) according to the manufacturer's instructions (Herr et al., 1995). The PCR product was detected by UV light in agarose gel with ethidium bromide and the embryos were scored as Y chromosome determinant positive (male) or Y chromosome determinant negative, respectively. All instruments represent a transferable unit that can be used in field conditions (AB Technology, Inc., Pullman, WA).

When the procedure was completed, i.e. about 3–3.5 h after cell sample isolation, the embryos were transferred. The results of PCR in HQ and LQ embryos were compared.

Embryo transfer

After sex diagnosis, the embryos were transferred ipsilaterally into synchronized recipient heifers (n = 174) of body weight 340–370 kg with physiological status of reproductive organs. Due to the unexpected difference between numbers of sexed embryos and available recipients, not all sexed embryos were transferred. Pregnancy diagnosis was performed ultrasonographically on Day 21 after transfer and confirmed by rectal palpation 7–21 days later. The sex of calves (n = 92) was confirmed after birth.

Statistical analysis

The obtained data were analyzed by the χ^2 test (2 × 2 contingency tables). The results of the analyses are summarized in Tables 1–3.

RESULTS

Table 1 summarizes the feasibility of sex determination with respect to the morphological quality of embryos. A total of 310 HQ embryos were investigated and sex analysis was successfully carried out in 275 cases (88.7%). On the other hand, the analysis of 170 LQ embryos was completed only in 111 instances (65.3%, $P < 0.01$). Pregnancy rates according to the morphological quality of sexed embryos are shown in Table 2. After the transfer of HQ embryos with determined sex, 56.6% of recipients became pregnant (69/122). The conception rate after ET of the sexed LQ embryos (27/52, 51.9%) was not significantly different. Table 3 compares the accuracy

Table 1. Sex determination in high and lower quality D7-D8 bovine embryos

Embryo quality	Biopsied embryos (*n*)	Completed sex determination		Questionable determination		Unsuccessful determination	
		n	%	*n*	%	*n*	%
High	310	275	88.7[a]	8	2.6[c]	27	8.7[e]
Lower	170	111	65.3[b]	35	20.6[d]	24	14.1[f]

a:b, c:d = $P < 0.01$, e:f = $P > 0.05$

Table 2. Pregnancy rates achieved after the ET of high and lower quality sexed D7-D8 bovine embryos

Embryo quality	Transferred embryos (*n*)	Pregnant animals	
		n	%
High	122	69	56.6[a]
Lower	52	27	51.9[b]

a:b = $P > 0.05$

Table 3. Sexing accuracy rates after the calving of recipients to which were transferred high and lower quality sexed D7-D8 bovine embryos

Embryo quality	Estimated as female (*n*)	Female calves born (*n*)	Male calves born (*n*)	Sexing accuracy rate (%)
High	66	63	3	95.5[a]
Lower	26	25	1	96.2[b]

a:b = $P > 0.05$

rates of sexing after calving between both embryo quality groups. After ET of the 66 HQ embryos, determined as females, 63 healthy female calves were born (95.5%). Similarly in the LQ embryo group, from 26 embryos determined as females, 25 female calves were born (96.2%).

DISCUSSION

Procedures based on the PCR amplification of specific DNA sequences of the Y chromosome used for the sex determination of preimplantation bovine embryos represent a good example of the rapid application of molecular biology techniques to the field. However, before this could be realised many of the steps of this technology had to be adapted. First of all, it was necessary to refine microsurgical intervention to enable the isolation of blastomeres from different developmental stages of embryos with minimal damage. The procedure may differ in details from laboratory to laboratory according to the skill and experience of the staff (Bredbacka et al., 1994; Thibier and Nibart, 1995; Shea, 1999; Yu et al., 2006). As described in more detail in the Material and Methods section we used for this purpose the twinner system (AB Technology, Inc., Pullman, WA), and similar to others we recorded only minimal harm to embryonic morphology after controlled operations under a stereomicroscope. Also, the randomly performed culture of treated and untreated embryos did not reveal differences in subsequent development.

Concomitantly with the possible harmful effects of microsurgery, the viability of embryos may be compromised by the reduction of cells after the removal of samples for sex analysis. It is known that a higher number of blastomeres in a sample increase the chances of determining the sex of progeny more accurately, but on the other hand, the artificial reduction of cell mass influences to a greater or lesser extent developmental ability. If it drops under a critical level the embryo dies. Naturally, the volume of isolated samples is closely related to the developmental stage of the experimental organism. Tominaga and Hamada (2004) isolated only

1 or 2 cells from 8–16 blastomeres embryos and sex was safely identified in nearly 90% instances. Moreover, the experimental and control embryos cleaved to blastocysts at the same rate (65.1% and 74.5%). Low number of cells, between 3–6, isolated from early cleaved stages have been reported also in papers published by Machaty et al. (1993), Taneja et al. (1998), Chrenek et al. (2001), Park et al. (2001), Lee et al. (2004), but little information is available about the real sex of progeny derived after the transfer of analysed embryos. From experimental aspects these data are certainly valuable, but field praxis requires exclusively embryos in more advanced developmental stages and Shea (1999) after the evaluation of numerous experiments concluded that the removal of 10–20% of embryonic mass for sex analyses had no detectable harmful effect on the pregnancy rate after transfer. Yu et al. (2006) also posits that larger samples are better for sex determination but that the viability of treated embryos may be compromised. This is particularly true when such embryos are subsequently exposed to freezing and thawing procedures (Lee et al., 2004). In our investigations we used embryos in similar developmental stages, but we isolated from HQ embryos only 6–12 cells and as the presented results demonstrate this amount was sufficient for safe analysis and, moreover, it can be expected that the integrity of the experimental organisms remains intact. As evident from Table 1, after the use of the described procedures sex can be defined in nearly 90% of HQ embryos, which is in full agreement with data (80–95%) reported by most other authors.

Present studies pay strong attention to those embryos classified as LQ because they represent a very valuable material for breeders and in are often observed in a high frequency in most flushings of superovulated donors. Table 1 demonstrates that they may also be included in sex determination technology. In contrast to HQ their cell number is lower and in addition some cells are expelled to the space under the *zona pellucida* or to the embryonic cavity and thus cannot participate in embryo development. When carefully removed, without damage to proper embryonic structures, they can serve as samples for sex analysis.

However, as it is clear from Table 1, not all of them are able to fulfil this expectation and in more than 20% of cases the result is problematic. The reason for the differences may be a progressive destruction of isolated cells from some embryos that are not optimal sources of DNA for analysis (Yu at al., 2006). But those embryos in which the sex is clearly defined are able after transfer to establish pregnancy at nearly the same rate as identically treated counterparts from HQ group. Very important appears to be the observation that treated embryos with clearly determined sex from the LQ group also induce comparable pregnancy after their transfer. This observation supports our previous studies where we concluded that the removal of cells localized in the perivitelline space may have a beneficial effect on subsequent development (Lopatarova et al., 2001). The authors suggested two hypotheses for such an effect. The first supposes that the separation of dead or dying cells is accompanied by the removal of the source of molecules that may exert deleterious effects on healthy embryonic mass. The second considers that the cutting of the *zona pellucida* in LQ embryos may assist at hatching as problems in this embryo class are sometimes due to a lower cell count which may reduce mechanical pressures generated by expanding embryo. Additional experiments are necessary to establish the accuracy of these hypotheses.

From the available reports it is evident that other approaches based on similar theoretical background can also be successfully used for the determination of sex in bovine embryos. One of them, the loop-mediated isothermal amplification (LAMP) is less time consuming and offers quite promising results with a high accuracy (Hirayama et al., 2004). At the same time there was published the paper describing how the high-titer rat H-Y antibody induced the developmental arrest of male embryos (Ramalho et al., 2004). This non-invasive embryo sexing requires further elucidation, however.

Our results summarise the observations obtained by the evaluation of a relatively high number of bovine embryos of different quality. The fate of each experimental object was carefully monitored after the completion of a single treatment in the laboratory and if selected embryos were transferred, particular attention was paid to the sex of progeny after parturition. All key parameters are comparable with those published by above mentioned authors but in addition our results show that LQ embryos are also suitable candidates for sex determination technology. It should be stressed that the proportion of embryos with defined sex is lower than in the HQ group, but that the pregnancy rate and accuracy of analyses are nearly identical (Table 2 and 3). Moreover, the remaining treated embryos with undefined sex are able after transfer

to develop to birth as untreated counterparts at a very similar rate. The application of these observations to the field increases chances of realising breeding programmes more rapidly and minimises the risk of a decrease in the number of calves from selected donors. Also, for operators these observations are promising from technical and economical aspects due to the fact that they may have a higher collection of embryos for analysis at a session.

Our results indicate that even such embryos together with their DNA isolated from blastomeres in the perivitelline space are, despite some limitations, suitable material for sex determination analysis. Moreover, the results obtained in field trials showed that the pregnancy rate in both groups was comparable and that therefore a broader spectrum of embryos is accessible for sex determination without a negative impact on reproductive parameters.

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