Acta vet. cand. 1991, *32,* 47-53.

Bisection of Bovine Morulae and Blastocysts from Superovulated Danish Dairy Cows

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Holm, P., T. Greve, A. Bak and M. Schmidt: Bisection of bovine morulae and blastocysts from superovulated Danish dairy cows. Acta vet. scand. 1991, 32, 47-53. - Sixtyfour compacted morulae and blastocysts were bisected with a microscalpel. The majority of the demi-embryos (n = 122) were reinserted into separate zona pellucidae (ZP) before non-surgical transfer to 113 syncronized recipients, as singles (n = 98) (DE-S) or in pairs (n = 30) (DE-P). Thirty non-manipulated embryos (E) were transferred during the same period and served as controls. Pregnancies were diagnosed by rectal palpation 4-7 weeks after transfer. The pregnancy rates for DE-S, DE-P and E were 32%, 53% and 40%, respectively ($P > 0.05$). A substantial number of abortions were recorded between 50 and 250 days of pregnancy among the recipients with DE-S. The fetal survival rate for DE-S was reduced to 21% and significantly lower ($p < 0.05$) than the survival rates of DE-P (43%) and E (40%). The quality of DE and the presence of ZP did not significantly influence the results. No conclusive reasons for the fetal loss could be found but different possibilities are discussed.

demi-embryos; pregnancy rates; fetal survival rates; abortions.

Introduction

As early as 1974 *Trounson & Moore* published results from an attempt to produce identical offspring from ovine embryos. It was *Willadsen* (1979), who developed a method with separation of blastomeres from precompacted embryos for producing identical offspring in sheep. This method has since been used with modifications in several studies which aimed at producing identical twins, triplets and quadruplets in farm animals as follows: Sheep: *Meinicke-Tillmann et al.* 1983, *Willadsen* 1980; cattle: *Willadsen et al.* 1981, *Willadsen & Polge* 1981; horses: *Allen & Pashen* 1984.

Simple methods for bisecting post-compaction bovine embryos with either a microscalpel (*Ozil* 1983, *Williams et al.* 1982) or a glassneedle (*Lambeth et al.* 1983, *Ozil et al.* 1982) have proved to be reliable and quick, and similar techniques have been adapted by many research groups and commercial ET-units in order to produce indentical twin calves and/or increase the number of pregnancies after embryo recovery and transfer. A prerequisite for practical application of bisection of embryos from farm animals is availability of a relatively simple splitting technique that can form an integrating part of the regular embryo transfer unit. Many recent studies of bisection of embryos have therefore aimed at further simplifying the methods, such as utilizing very simple techniques (*Rorie et al.* 1985, *Williams & Moore* 1988) or transferring bisected post-compaction embryos without zona pellucida (*Che-*

sné et al. 1987, *Warfield et al.* 1986, *Willadsen & Godke* 1984).

The aim of this study was to produce identical twin calves by means of bisection of post-compaction embryos recovered by non-surgical techniques from Danish dairy cows.

Materials and methods

Embryos were collected non surgically from superovulated dairy cows and heifers 6½ to 7 days after estrus (*Greve* 1981). A modified Dulbecco's phosphate buffered saline (PBS) (*Whittingham* 1971) was used as flushing medium. When used for holding, micromanipulation and transfer of embryos, PBS was supplemented with 20% heat treated fetal calf serum, and then sterile filtered. (1) Only compact morulae (CM) and blastocysts (YB: young blastocysts & XB: expanded blastocysts), graded as excellent or good (*Lindner & Wright* 1983), were used for micromanipulation.

The bisections were done with 2 Leitz micromanipulators under a compound microscope (Laborlux 2, Leitz) at 100x magnification. The bisection procedure was modified after *Picard et al.* (1986) as earlier described in details by *Jørgensen et al.* (1985). It involves opening of zona pellucidae and bisecting the embryonic cell mass outside the original zona pellucida with a triangular microscalpel against the bottom of a pertri dish. The microscalpel was produced from breakable razor blades (Storz, Germany) and glued onto a capillary glass pipet.

After splitting the demi-embryos were grouped into 4 classes (class 1 being the best). The classification was based on (a) the relative size of the demiembryo, (b) the cellular appearance and (c) the integrity of the bisected cell mass. All demiembryos, except 6, were reinserted into either the original zona pellucida or a surrogate zona prepared from oocytes or degenerated/retarded embryos. The demiembryos were left in the holding medium at room temperature until transfer, which took place within 2-4 h after the microsurgical intervention.

A total of 128 demi-embryos produced from 64 embryos were transferred non surgically to 113 heat-synchronized heifers (n = 90) and cows (n = 23).

Ninety-eight (98) recipients (81 heifers), received a single demi-embryo (DE-S) in the distal third of horn ipsilateral to the corpus luteum and 15 recipients (all but 1 cow) received a pair of demi-embryos (DE-P) in the posterior part of the horn ipsilateral the corpus luteum. A total of 30 whole embryos (E) transferred to synchronized recipients during the same period estrous served as controls. Pregnancies were diagnosed by rectal palpation 5-7 weeks after transfer.

Results

Pregnancy results after transfers of DE-S, DE-P and E appear in Table 1.

Pregnancy rate

The highest pregnancy rate (53%) was obtained by transferring 15 DE-P, but this rate was not significantly different from the rates observed after transfer of DE-S (32%) or E (40%). Fetal survival rate and abortion: The percentage of calves born (fetal survival rate) after transfer of DE-S (21%) was significantly lower ($p < 0.05$) than those obtained by transferring DE-P (43%) as well as E (37%). An important reason for this is, that among the recipient heifers in the major herd, which all received DE-S, 4 *aborted* between the 60th and 90th day of gestation, and 4 between the 173th and 246th day of gestation. Contrary to this, none of the recipients receiving DE-P aborted, and only 1 out of the 12 pregnant recipients aborted after having received E. No macroscopic abnormalities were found in the latter 4 late aborted fe-

Table 1. Pregnancy rates and fetal survival rates after transfer of demi-embryos as singles (DE-S), pairs (DE-P) or as non-manipulated embryos (E) to estrous synchronized recipients.

Embryos transferred		N° of demi-emb. <DE-pair>	N° of preg. recipients (%)	N° of calves born (% of DE[1])	Pairs of twins (% twinning)
DE-S	CM	40 <20>	13 (31)	9 (23[2])	3 (15)
	YB	42 <21>	15 (36)	9[3] (23)	0
	XB	16 < 8>	3 (19)	2 (13)	0
Total		98 <49>	31 (32)	20[ab] (21[4])	3[c] (6)
DE-P	CM	22 <11>	6 (55)	8 (37)	3 (27)
	YB	8 < 5>	2 (50)	4 (50)	2 (50)
	XB	-	-	-	-
Total		30 <16>	8 (53)	12[a] (43)	5[c] (33)
DE + pair		128 <64>	39 (35)	32 (65)	8 (13)
E	CM	18	7 (39)	6 (33)	-
	YB	7	2 (43)	6 (43)	-
	XB	5			
Total		30	12 (40)	11[b] (37)	-

Numbers with same notations (a, b or c) are significantly different (0,02 < P < 0,05).

(1) Recipients that were culled before calving and abortions are excluded from the survival rates.

(2) One pregnant recipient culled. Rae calculated from 39 DE transferred to same number of recipients.

(3) Two pregnant recipients bearing a set of twins were culled. Rate calculation from 40 DE transferred to same number of recipients.

(4) Rates calculated from 95 DE in 95 recipients, see notes 1, 2, 3.

(5) Rates calculted from 125 DE inl 10 recipients, see notes 1, 2, 3.

tuses, and one of these (aborted day 173 after transfer) was bacteriologically and virologically examined, but no conclusive diagnoses could be made. None of the 4 fetuses aborted within 90 days were examined.

Embryo quality

Fiftytwo recipients received a single demi-embryo classified in class 1, 36 received a class 2 demi-embryo, and 10 received a class 3 demi-embryo. Of these recipients, 16 (31%), 14 (39%) and 1 (10%), respectively, became pregnant, but these differences were not significant. Of the pregnant recipients in the respective groups, 15, 12 and 1 were allowed to go to term. Abortions occured in 2 (13%) of the recipients carrying a class 1 demi-embryo, and in 6 (50%) of those carrying class 2 demi-embryos, while none in group 3 aborted.

Embryo stage

The embryo stage did not influence the pregnancy raes, and there was no significant difference in the results after separate transfer of demi-embryos in the original zona pellucida (n = 51), in a surrogate zona pellucida (n = 41) or without zona (n = 6), as 14 (28%), 15 (37%) and 2 (33%) recipients, respectively, became pregnant. The pregnancy rate per bisected embryo tended to be higher (NS) compared to whole embryos, as 39 recipients

were pregnant after transfer of 64 bisected embryos (61%) versus 12 recipients pregnant after transfer of 40 whole embryos (40%).

Discussion

In this study the overall pregnancy rate after transfer of demi-embryos did not differ significantly from the pregnancy rate obtained by transferring whole embryos (35% versus 40%). This is similar to other studies, reporting pregnancy rates of 17-82% for non-surgical transfer of DE-S and/or DE-P (*Arave et al.* 1987, *Baker & Shea* 1985, *Brem et al.* 1985, *Lambeth et al.* 1983, *Leibo & Rall* 1987, *Ozil* 1983, *Ozil et al.* 1982, *Picard et al.* 1986, *Takeda et al.* 1986, *Voelkel et al.* 1984, *Williams et al.* 1984).

The number of calves born and the number of twins were significantly higher when the half embryos were transferred in pairs compared to singles. Similar improvement of results (pregnancy, fetal survival and twinning rates) has been reported by *Lambeth et al.* (1983) and *Ozil* (1983). One reason for this improvement could be that the production of early pregnancy factors from a single demi-embryo is not sufficient to prevent luteolysis of the corpus luteum (*Thatcher et al.* 1988), bearing in mind that the cell number of the individual demi-embryo is more than halved, as the mechanical destruction of cells during bisection amounts to 10-20% (*Jørgensen et al.* 1985). Therefore, when transferring demi-embryos in pairs the total embryonic response per se will be significantly increased.

The fetal survival rate was negatively affected by the high number of abortions in the major herd. Eight (29%) out of 28 recipient heifers that were not culled aborted between day 60 and day 246. Normally, most embryonic losses in embryo transfer occur before implantation (*Heyman* 1985) and our finding is in contrast to other reports of abortion rates (3-6% after transfer of demi-embryos, *Arave et al.* 1987, *Williams et al.* 1984). Only *Brem et al.* (1983) and *Willadsen & Polge* (1981) have reported similar large numbers of abortions after day 50 following transfer of manipulated (half and quarter, respectively) embryos, and 25% foetal losses between day 45 and 90 has been reported (*Heyman* 1985).

The extent and cause of this phenomenon needs further detailed investigations, but some speculations can be made. Low-grade uterine bacterial or mycotic infections induced by transfer cannot be excluded, but it is unlikely in this study, as none of the examined fetuses showed any signs of these types of infections.

Abortions caused by virus, eg. BVD-virus, is a more likely possibility, as all abortions were found among the heifers in one particular herd. BVD-virus is known as the most important cause of abortions in cattle in Denmark (*Meyling* pers. comm.), and in 1 fetus examined, BVD-like immunoflourescence reactions were found in the brain tissue. The fact that the abortion frequency of the class 2 demi-embryos was higher than among the class 1 demi-embryos may also be part of the reason, but the numbers are too small to be conclusive. Others have clearly stated that the highest classified demi-embryos have the highest viability in vivo (*Arave et al.* 1987, *Voelkel et al.* 1984) and in vitro (*Picard et al.* 1986), but the quality has not been reported to affect the survival rate of the embryos after 60 days of pregnancy. *Willadsen & Polge* (1984) suggested that many abortions which occurred after day 50 in their study might

reflect a dysgenesis of the transferred embryos due to a small number of cells in the inner cell mass.

The same could be the case with the class 2 embryos. Further, *Philipsen* (1956) described several pregnancies, in which the allantochorionic membranes continued to grow until 2½-3 months of gestation, although the amnionic cavity of the embryo had been manually destroyed prior to day 45 of pregnancy. Such pregnancies are easily mistaken as normal in routine rectal examination (*Philipsen & Sørensen* 1972), and it cannot be ruled out that some of the earlier abortions may be of this type.

Transfer of 6 demi-embryos without zona pellucida resulted in birth of 2 calves. This indicates that the role of the zona pellucida seems to be of minor importance in post-compaction embryos. Similar conclusions have been reached by others in sheep (*Trounson & Moore* 1974, *Willadsen & Godke* 1984) and cattle (*Voelkel et al.* 1984, *Williams & Moore* 1988). The trophoblastic layer forms an epithelium-like surface of the post-compaction embryos, and it is seen to regenerate very shortly after bisection (*Jørgensen et al.* 1985, *Ozil* 1983), thus protecting the bisected inner cell mass from the uterine environment.

In conclusion, this study has shown that bisection of bovine embryos can be incorporated into the regular embryo transfer routine and thereby increase the potentials, since the total number of calves per embryo is increased. More specifically the study indicated that transfer of demi-embryos in pairs results in both higher pregnancy and fetal survival rates than single transfers, and that the abortion incidences reached a substantial level in the group of transfer of a single demi-embryo.

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Sammendrag

Bisektion of morula og blastocyststadier fra superovulerede køer.

Fireogtres (64) kompakte morulae og blastocyster blev kløvet med en miroskalpel. Hovedparten (n = 122) af demiembryonerne (DE) blev genindsat i separate zonae pellucidae (ZP), inden de blev transplanteret ikke-kirurgisk, enten enkeltvis (DE-S, n = 98) eller parvis (DE-P, n = 30) til 113 synkroniserede recipienter. Tredive (30) hele embryoner (E), som blev transplanteret indenfor samme periode, udgjorde kontrol gruppen. Drægtighed blev diagnosticeret ved rektal palpation 5-7 uger efter transplantationen. Drægtighedsprocenterne for DE-S, DE-P og E var henholdsvis 32%, 55% og 40% ($P > 0,05$). Et væsentlig antal aborter blev iagttaget mellem 60. og 250. drægtighedsdag blandt DE-S, således at den føtale overlevelsesrate hos denne gruppe kun blev 21%, hvilket var signifikant lavere ($P < 00.5$) end overlevelsesraterne for DE-P (43%) og E (37%). Kvaliteten af DE og tilstedeværelsen af ZP påvirkede ikke resultaterne signifikant. Der blev ikke fundet konklusive årsager til aborterne, men forskellige muligheder diskuteres.

(Received March 2, 1990; accepted March 23, 1990).

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