Generation of identical twin piglets following bisection of embryos at the morula and blastocyst stage

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Porcine morulae and blastocysts were microsurgically bisected and the resulting zona pellucida-free demi-embryos were either cultured *in vitro* for 48 h or transferred after 24 h of culture into – 24 h asynchronous recipients. All demi-embryos were evaluated according to morphological criteria and classified into three categories (excellent, fair or degenerated). The average diameter and the number of cells were determined. Of 1162 bisected embryos, 764 pairs (66%) were evaluated as transferable after 24 h of culture *in vitro*. The average diameter after 48 h of culture *in vitro* was different ($P < 0.01$) among demi-embryos of the three morphological categories as was the number of cells. The greatest diameter and the greatest number of cells were found in demi-embryos classified as morphologically excellent. A total of 22 of 27 recipients (81.5%) remained pregnant and 21 recipients delivered 126 piglets of which six were stillborn. The survival rate of demi-embryos in farrowing recipients was 21.2% (126 of 594). Litter size was significantly reduced in recipients after transfer of demi-embryos compared with that of mated controls (6.0 ± 2.5 versus 10.8 ± 2.1 piglets). Similarly, the *postpartum* losses of piglets were higher in the experimental than in the control gilts (26.7% versus 11.6%). Duration of gestation, average birth weight and daily weight gain were not affected. Among the 126 piglets, seven pairs of identical twins (2.3% of 311 transferred pairs) were identified using several genetic markers in blood (blood groups, polymorphic enzymes and plasma proteins) in a total of 25 gene loci. DNA fingerprinting revealed an identical banding pattern between the two partners of each of the seven pairs. Birth and weaning weight as well as daily weight gain varied considerably between monozygotic partners.

Introduction

In contrast to the well advanced embryo bisection technology in cattle (Ozil, 1983; Williams *et al.*, 1984; Arave *et al.*, 1987; Seike *et al.*, 1989; McEvoy and Sreenan, 1990; Gray *et al.*, 1991; Kippax *et al.*, 1991) and small ruminants (Gatica *et al.*, 1984; Willadsen and Godke, 1984; Tsunoda *et al.*, 1985; Chesné *et al.*, 1987; Udy, 1987; Shelton and Széll, 1988; Széll and Hudson, 1991; Shelton, 1992) studies in pigs are scarce and very few sets of identical twin piglets have been obtained upon transfer of bisected pig embryos (Polge, 1985; Nagashima *et al.*, 1988; Ash *et al.*, 1989). The main obstacles to the generation of identical twin piglets are inherent to some characteristics of reproductive physiology of pigs. Pregnancy is only maintained in pigs when a minimum of 2–4 embryos implant and at least 25% of the uterine surface is covered by embryonic tissue (Polge *et al.*, 1966; Webel *et al.*, 1970). Furthermore, the transuterine migration during the preimplantation phase (Dziuk *et al.*, 1964) does not allow a separate transfer of demi-embryos into each uterine horn, which complicates identification of monozygosity. Identification of monozygosity requires the

analysis of genetic polymorphisms using genetic markers (blood groups, polymorphic enzymes, plasma proteins) for which established procedures are available (Hesselholt, 1966; Schmid and Buschmann, 1985; Juneja and Gahne, 1987). These methods are routinely used to characterize the genetic identity of individual animals. DNA-fingerprinting has been introduced to test monozygosity (Pemberton and Amos, 1990). The procedure is based on the observation that in many organisms short repeated DNA sequences are found at numerous positions in the genome. The basic unit of these simple repetitive DNA sequences is a few base pairs that are located in the noncoding regions between genes. The number of repeats of the simple sequences and the restriction enzyme sites in the noncoding regions are highly variable and can therefore be used to identify the genetic identity of an individual (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987). A reliable procedure for generation and identification of identical twin piglets would be valuable for both the employment of new breeding strategies and for basic biomedical research.

The purpose of our investigation was to test the efficiency of obtaining monozygotic twin piglets following microsurgical bisection of morula and blastocyst stages and to elucidate the suitability of DNA-fingerprinting for improving identification of identical twins among the piglets that are born.

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Materials and Methods

Animals and embryos

Prepubertal German Landrace gilts (182–246 days of age, 78–123 kg bodyweight) were used either as donors or as recipients. Superovulation was induced by i.m. injections of 1500 iu pregnant mares' serum gonadotrophin (PMSG: Inter-gonan; Vemie, Kempen) followed 72 h later by an i.m. injection of 500 iu hCG (Ekluton, Vemie, Kempen). Donors were mated to a fertile German Landrace boar 24 and 48 h after administration of hCG. When appropriate boars were not available, semen was collected and the donors were inseminated with 100 ml of at least 2×10^9 freshly diluted spermatozoa. Blood samples were collected from the cava anterior vein of donors for determination of genetic parentage. The day of the first mating or insemination was designated as day 0. The recipients underwent a parallel treatment (800 iu PMSG, Inter-gonan), but 24 h behind the schedule of the donors, making them – 24 h asynchronous to the donors. Oestrus was detected using test boars and only females showing clear signs of oestrus and accepting the boar were used as potential recipients. Three to four donors (in total 103) were used per experimental day and at the same time two to three recipients (in total 32) were prepared. The selection and mating of the donors for an experimental day took into account the fact that the parents of the potential embryos to be bisected were not related to each other, to facilitate the determination of parentage and monozygosity. All matings or inseminations of one particular donor were done by the same boar. There were no siblings among the donors and boars assigned to an experimental day. Donors were killed on day 5 at the Institute's abattoir and the genital tract was removed immediately. At the same time, blood samples were taken for tests of parentage and monozygosity. Within 5 to 10 min after killing the animals, the uterine horns were flushed using standardized procedures and the morphological quality of the collected embryos was assessed (Niemann *et al.*, 1983a; Niemann and Elsaesser, 1987). Subsequently, morulae and blastocysts classified as excellent or good from one individual donor animal were placed into 2 ml Dulbecco's phosphate-buffered saline (PBS – Sigma, St Louis, MO) supplemented with 10% newborn calf serum (NBCS, Boehringer, Mannheim) at 37°C in a humidified atmosphere with 5% CO₂ in air until being used for microsurgical bisection.

Microsurgical bisection of pig embryos

The microsurgical bisection was performed at room temperature using a micromanipulation unit consisting of an inverted microscope (Diavert, Leitz, Wetzlar) and a mechanical micromanipulator (Leitz) which was placed on the right side of the microscope. All embryos were bisected using a sterile microsurgical blade (Spezialklinge S, Heidelberg) which was fixed with a special blade holder (Frohnhäuser, Munich) without a holding pipette. Bisection was done under a microscope at $\times 100$ magnification in a droplet of medium (PBS supplemented with 10% NBCS) covered with silicone oil. The embryos were fixed during bisection by scratching the bottom of the plastic Petri dish with the microblade. The microblade

was held above the embryo placed between two scratches parallel to the blade. The microblade was brought slowly down and the embryos were bisected and the zona pellucida was cut into two pieces. The inner cell mass of blastocysts was carefully separated into two halves. All demi-embryos were cultured or transferred without zona pellucida. Only one embryo was bisected per droplet of medium and each Petri dish contained three droplets of medium. It was thus possible to split three embryos successively within approximately 10 min. Immediately after bisection all demi-embryos were transferred into culture medium.

In vitro culture of demi- and intact embryos

A representative number of nonbisected embryos from each donor animal was cultured as controls. These were approximately 20–25% of the embryos randomly collected at an experimental day, at the same stage of development and of the same morphological quality score as the embryos used for bisection. The culture medium was Krebs–Ringer–bicarbonate solution (KRB) (Davis and Day, 1978) modified (mKRB) according to Saito and Niemann (1991); mKRB lacked lactate and pyruvate but contained EDTA and contained a higher concentration of NaCl (1.168 g l^{-1}). The mKRB solution was supplemented with 10% heat-inactivated lamb serum (Seromed, Berlin; heat inactivated for 30 min at 56°C), sterile filtered and stored at 4°C for a maximum of 7–10 days. Only one pair of demi-embryos was cultivated per Petri dish at 37°C in a humidified atmosphere with 5% CO₂ in air (Niemann and Elsaesser, 1987), for either 24 h when they were to be transferred to recipients or a total of 48 h when development *in vitro* only was studied (Table 1). Embryos used exclusively for *in vitro* culture were collected on 5 experimental days. In addition, demi-embryos collected for transfer and not used for this purpose were similarly cultured *in vitro*.

The development of the demi-embryos was evaluated after 24 h using morphological criteria such as rounding up of the halves, formation of a blastocoel and the incidence of excluded degenerated cells. All halves were classified into three groups according to Nagashima *et al.* (1989): category I: typical blastocysts with very few degenerated cells (<10%) and a clearly visible inner cell mass (excellent); category II: blastocysts of a smaller diameter than group I with a larger number of degenerated cells (10–20%) and the inner cell mass not clearly visible (fair); category III: small blastocysts without a visible inner cell mass and a large number of degenerated cells (>20%) or some enlarged nonintegrated blastomeres or completely degenerated halves (degenerated).

A pair of demi-embryos was evaluated as being transferable only when both halves were classified at least into category II.

Transfer of demi-embryos

In cases in which too few transferable pairs (in total 394) were available, single halves ($n = 22$) classified into category I were cotransferred to increase the probability of maintaining pregnancy. In total, 810 halves were washed twice in PBS

Table 1. Classification of pairs of pig demi-embryos following 24 h of culture *in vitro* and their distribution in *in vitro* and *in vivo* experiments

Classification	I/I	I/II	II/II	I/III	II/III	III/III	Total
<i>n</i>	332	320	112	170	143	85	1162
%	29	27	10	15	12	7	
<i>In vitro</i> culture							
<i>n</i>	85	205	80	146*	143	85	
%**	11	27	11	—	—	—	
Transfer							
<i>n</i>	247	115	32	22*	—	—	
%**	32	15	4	—	—	—	

I = excellent morphology; II = good morphology; III = degenerated.

Only demi-embryos in categories I and II were considered as transferable. *A total of 22 single demi-embryos were transferred.

**Percentage levels related to transferable embryos only.

supplemented with 5% NBCS and were then transported at 37°C to the site of transfer approximately 8 km from the laboratory. Food was withheld for 24 h before the surgical intervention in the recipients. Anaesthesia was induced by an i.m. injection of 200 mg Azaperon (Stresnil, Jansen, Neuss) followed 20 min later by an i.v. injection of Metomidathydrochlorid (Hypnodil, Jansen, Neuss) into the ear vein at 500–1000 mg per animal to ensure the onset of a suitable anaesthetic stage. The abdominal cavity was opened in the linea alba, and the ovaries were exposed and checked for the presence of a sufficient number of corpora lutea. Transfers were performed only when at least six corpora lutea could be detected. A 1 ml syringe was used to suck the demi-embryos with the least transfer medium possible into a sterile transfer catheter (Ernährungssonde Braun, Melsungen). The uterine wall of one horn was punctured by a blunt metal needle and the transfer catheter was inserted approximately 2–3 cm into the uterine lumen where the demi-embryos were deposited. The abdominal cavity was then closed using standardized surgical procedures and all animals received a prophylactic penicillin–streptomycin treatment. All recipients were checked for signs of oestrus at the next expected day of oestrus by inspection of the vulva and testing with a boar. At about day 45 after transfer, the animals underwent an ultrasound examination (Picker CS 9000 with a 5 MHz probe). Recipients that did not show signs of oestrus and in which fetuses could be detected were considered pregnant. Blood samples were collected from all piglets at approximately 4–6 weeks of age to test for monozygosity. This schedule for collecting blood samples was necessary as the various electrophoretic analyses required a total of 15 ml blood and we had previously found that collection of 10–15 ml blood from newborn offspring interfered with the health and subsequent development of the piglet.

The further development of the piglets derived from transfer of demi-embryos was compared using 20 gilts (controls) that had been mated at their first oestrus to boars involved in this study. Both the recipients and controls were randomly selected during the same period from the same pool of animals in our experimental herd and were of the same age and bodyweight.

Determination of diameter and number of cells in demi-embryos developed in vitro

After culture *in vitro* the diameter and number of cells were determined from a representative percentage (6–14%) of embryos randomly selected from controls and demi-embryos in each morphological category. The diameter was measured at a magnification of $\times 100$ (Axiovert 35 M, Zeiss, Oberkochen) using a micrometer. The zona pellucida was removed by a short-term incubation in a 1% pronase solution (Protease: Sigma) in control embryos that had not hatched from the zona pellucida at the end of the culture period. Subsequently halves and controls were prepared for counting the number of nuclei as described by Tarkowski (1966). After hypotonic treatment, fixation and drying at room temperature, all preparations were stained with a 10% Giemsa solution. The stained nuclei were counted at a magnification of $\times 250$ (M20; Wildt, Heerbrugg).

Determination of monozygosity

Each recipient received demi-embryos from a maximum of four different genetic parents. The parentage of piglets was determined by typing the genetic parents and piglets according to blood genetic markers (blood groups, plasma proteins and polymorphic enzymes). Monozygotic twins could be identified by comparing the various parameters among piglets of the same sex within one litter. Blood was stabilized in a solution of 2% sodium citrate, 0.5% sodium chloride and 0.06% sodium cyanamide in double-distilled water using agglutination and haemolysis procedures (Schmid and Buschmann, 1985; Vögeli, 1990) and 15 blood group systems involving 58 factors were determined. The serum and enzyme systems were determined electrophoretically; the AMY- and TF-system in starch gel, the GPI-, PGD-, PGM- and ADA-system in an agarose gel (Verhorst, 1973), the A1BG-, PI-1, PI-2 and PI-3-system were analysed by two-dimensional electrophoresis (Juneja and Gahne, 1987). In total, 25 gene loci were involved.

Blood samples from piglets that were identified as identical twins by blood genetic markers underwent a DNA-fingerprinting procedure to confirm their genetic identity. Approximately 20 ml blood was collected, treated

Fig. 1. A pair of pig demi-blastocysts after culture *in vitro* for 24 h; both are classified as excellent (I/I). The inner cell mass is visible and only very few degenerated cells are apparent. Scale bar represents 25 μ m.

Fig. 2. A pair of pig demi-embryos after culture *in vitro* for 24 h; both are classified as fair (II/II). The inner cell mass is not clearly visible and a few degenerated cells are adhering to the blastocoel. Scale bar represents 25 μ m.

with EDTA and then stored frozen at -20°C . For DNA-fingerprinting, the DNA was extracted (Miller *et al.*, 1988) and digested with the restriction enzyme Hinf I. All hybridizations were performed with the ^{32}P -radiolabelled probe (GGAT)₄ (Epplen and Zischler, 1991; Schwaiger *et al.*, 1992).

Statistical analysis

Normal distributed variables such as diameters of embryos and birth weights of the piglets were analysed by the general least-squares method (GLM) (Searle, 1971). Random test comparisons were calculated with Student's *t* test for significant differences. Qualitative traits that were discontinuously distributed as binominal or multinominal data (Grizzle *et al.*, 1969) were analysed using the nonparametric Catmod procedure of the SAS statistical package (SAS version 6.04, 1989). The data are presented as means $\pm$ SD. Differences were considered to be significant at $P < 0.05$.

Results

From 91 donors an average of 42.1 ± 18.2 corpora lutea were obtained yielding 3017 ova and embryos and total recovery rate was 78.8% (33.2 ± 17.7 ova and embryos per donor). Fertilization rate was 82.2% and of the 2481 fertilized ova 2236 (90.1%) were classified as viable and were at the compacted morula to expanded blastocyst stage. A total of 1162 embryos, including 172 compacted morulae, 496 early blastocysts and 494 blastocysts, were microsurgically bisected in 27 experimental replicates.

Morphology of pig demi-embryos after 24 and 48 h of culture in vitro

Sixty-six per cent (764) of the 1162 pairs of demi-embryos were considered transferable after 24 h of culture *in vitro* (Table 1; Figs 1–3). The pairs that were not transferred to recipients underwent continuous culture for a further 24 h

Fig. 3. A pair of pig demi-blastocysts after culture *in vitro* for 24 h; one-half is classified as excellent, the other as degenerated (I/III). Scale bar represents 25 μ m.

before being evaluated again. In 61.6% of the pairs, the morphological classification was not altered compared with the preceding evaluation. In 18.8%, one-half received a better grade than 24 h before, and in 18.2% one-half had an impaired morphology. In 8 pairs (1.3%) one-half showed an improved, whereas the other half displayed an impaired, morphology compared with the first evaluation after 24 h of culture *in vitro*. The percentage of transferable pairs was different ($P < 0.05$) among blastocysts (71%), early blastocysts (65%) and morulae (53%).

Diameter of demi-embryos after culture in vitro for 48 h

The average diameter of demi-embryos classified in category I was greater ($P < 0.01$) than that of demi-embryos of categories II and III. The diameter of embryos in category II and III demi-embryos was also different ($P < 0.01$). The average diameter in control embryos was similar to that in demi-embryos of category I (Table 2). When considering the

Table 2. Average diameter (μm) of porcine demi-embryos and controls after culture *in vitro* for 48 h

Category	n	Mean	SD	Minimum	Maximum
I	70	210.6 ^a	33.1	150	290
II	77	161.6 ^b	25.7	110	220
III	29	121.7 ^c	25.6	70	161
Controls	42	213.7 ^a	25.2	170	280

I = excellent morphology; II = good morphology; III = degenerated. ^{a,b,c}Values with different superscript letters are significantly different ($P < 0.01$).

Table 3. Average number of cells in pig demi-embryos and controls after culture *in vitro* for 48 h

Category	n	Mean	SD	Minimum	Maximum
I	99	54.0 ^a	18.5	22	114
II	93	25.7 ^b	8.6	10	56
III	41	14.3 ^c	6.7	6	36
Controls	67	81.8 ^d	28.6	41	139

I = excellent morphology; II = good morphology; III = degenerated. ^{a,b,c,d}Values with different superscript letters are significantly different ($P < 0.01$).

average diameter in halves of categories I and II in relation to the classification of the respective partner, it became evident that, even when the two halves were classified into the same category, they were not the same size. The difference between halves both classified in category I was 19.4 μm and in halves both classified in category II 14.6 μm ($P < 0.05$).

Number of cells in demi-embryos after culture *in vitro* for 48 h

The average number of nuclei was highest in demi-embryos classified in category I and was different ($P < 0.01$) from that of halves in categories II and III, as was the difference between demi-embryos in categories II and III ($P < 0.01$). The average number of nuclei in control embryos was higher ($P < 0.01$) than that in demi-embryos of category I (Table 3). When the average number of nuclei was considered in relation to the respective partner, there were no differences in demi-embryos classified into category II. However, the number of cells in demi-embryos both classified in category I was different ($P < 0.01$) between partners (36 versus 54). Similarly, halves classified into category I possessed a greater number of cells when the respective partner was classified into category II or III (58 and 69 nuclei, respectively). The number of cells and the average diameter were closely correlated ($r = +0.85$).

Development of pig demi-embryos *in vivo*

Because of the technical difficulties in collecting larger numbers of zona-free blastocysts in a very small amount of

Table 4. Fertility parameters in gilts delivering piglets derived from demi-embryos or after mating (controls)

Parameter	Experimental animals	Controls
Duration of gestation (days)	115.5 $\pm$ 2.3	114.7 $\pm$ 1.3
Litter size	6.0 $\pm$ 2.5 ^a	10.8 $\pm$ 2.0 ^b
Birth weight (kg per piglet)	1.1 $\pm$ 0.4	1.3 $\pm$ 0.2
Daily weight gain (g)	228.2 $\pm$ 43.6	209.1 $\pm$ 39.5

^{a,b}Values with different superscript letters are significantly different ($P < 0.01$).

medium in the transfer catheter, only 759 demi-embryos were transferred corresponding to approximately 28 halves per recipient. A total of 22 of 27 recipients did not show signs of oestrus and were found to be pregnant at ultrasound scanning (81.5%). In total, 21 recipients (77.8%) farrowed and in 20 of these parturition occurred spontaneously and without problems. On day 120 after transfer, one recipient received an i.m. injection of a prostaglandin-analogue (250 μg Estrumate: Pitman-Moore, Grossburgwedel) to induce parturition and a stillborn piglet was delivered 20 h later. In total, 126 piglets were born of which 120 were alive. When the number of piglets born was related to the number of transferred halves in all 27 recipients, 16.6% (126 of 759) developed to piglets; when related only to the 21 farrowing recipients this percentage was 21.2% (126 of 594).

Litter size differed ($P < 0.01$) between the recipients and control animals, whereas duration of gestation, average birth weight and the daily weight gain were similar among the piglets of the two groups (Table 4). Of the 126 piglets, 70 (55.6%) were male and 56 (44.6%) were female. The corresponding figures for the 216 control piglets were 61.6% ($n = 133$) and 38.4% ($n = 83$). The recipients delivered a total of six dead piglets (five female, one male) (4.8%), whereas the control animals delivered only one dead male piglet (0.5%). The losses up to weaning were 26.7% (32 of 120) in piglets derived from demi-embryos and 11.6% (25 of 215) in the controls ($P < 0.05$). Approximately 72% of these piglets died because of insufficient development or because of maltreatment by the foster mother; the corresponding percentage for the controls was 88% (22 of 25). Eight piglets (6.4%) derived from the demi-embryos displayed anomalies such as cryptorchism, atresia ani, hernia formation and ventral cleft formation, compared with six (2.8%) control piglets; the difference was not significant.

Determination of parentage and monozygosity

In 93 of 120 piglets born alive (51 males and 42 females), blood samples were collected to determine parentage and monozygosity; the remaining 17 males and 8 females died before blood samples could be collected. Blood samples were not collected from one male and one female piglet as they had only one sibling of the opposite sex and therefore could not have a monozygotic twin partner. When using the blood genetic markers, 86 piglets could be related to their genetic parents; in five offspring within a litter of nine piglets, this was

Fig. 4. Banding pattern of a set of identical twin piglets (A and B) and of two nonrelated animals (C and D). The twins have an identical banding pattern, whereas the other siblings do not share common bands in all positions. Only the section with the best visible bands is shown.

not possible. Seven pairs of monozygotic twin piglets were identified. The monozygosity was confirmed in all pairs by DNA-fingerprinting, as all loci contained identical bands (Fig. 4). The seven pairs of identical twins had a different birth weight (60–320 g difference) and weaning weight (400–5800 g difference) as well as a different daily weight gain (10–186 g difference) (Table 5).

Discussion

The results of the investigation reported here demonstrate that pig demi-embryos possess a great developmental potential *in vitro* but their development *in vivo* is impaired compared with that of intact embryos. The level of blastocyst development *in vitro* (79%) in our experiments compared favourably with that reported by Nagashima *et al.* (1989), who obtained a

Table 5. Birth weight, weaning weight and daily weight gain in monozygotic piglets

Sex	Birth weight (g)	Weaning weight (kg)	Daily weight gain (g)
Female	1140	9.8	241
	1360	10.4	251
Female	1500	9.4	214
	1620	10.2	232
Male	1340	9.2	207
	1440	10.3	233
Male	1600	5.5	122
	1460	11.3	308
Female	1160	10.1	224
	1340	13.1	294
Male	1120	10.0	222
	1180	9.6	211
Female	1160	9.9	243
	840	9.0	227

higher percentage of transferable pairs of demi-embryos (85%) than in our experiments (66%). This higher percentage could be attributed to the different bisection technology used. However, the percentage of pairs of demi-embryos classified into category I was similar in both of these studies. The diameter and the number of cells in demi-embryos of the three morphological categories reflected the morphological evaluation of the halves. The close correlation between diameter and number of cells allows the size of the embryo to be used as a major criterion for the evaluation of pig demi-embryos after culture *in vitro*. Saito *et al.* (1991) reported that the average diameter of pig demi-embryos derived from bisected morulae or early blastocysts after 72 to 96 h of culture *in vitro* was higher than that found in the study reported here (288–310 µm, 95–126 cells).

The relative number of cells in demi-embryos, for example the average number of cells of demi-embryos in relation to that of the control embryos, can be used as an appropriate parameter to evaluate the developmental potential of demi-embryos. In cows, the relative number of cells in demi-blastocysts derived from early or late morulae was exactly 50% (Bredbacka *et al.*, 1991). We found a relative number of cells of 18%, 31% and 66% in categories III, II and I demi-embryos, respectively. If the loss of 10–15% cells caused by a microsurgical bisection (Skrzysowska and Smorag, 1989) is taken into account, demi-embryos classified in category I possess a high regeneration potential. This is underlined by the finding that the average diameter of these demi-embryos is almost identical to that of the control embryos. Halves of a particular pair were occasionally not of the same size (categories I and II) and number of cells (category I), even when they were classified into the same morphological category. Picard *et al.* (1986) reported a similar observation for cows with a mean difference of 23 blastomeres. These above findings clearly show the difficulty in splitting embryos in such a way that both halves contain an even number of viable cells and illustrates that some embryos were divided into two unequal parts. However, the percentage of cells destroyed during bisection

could have been greater in one half than in the other without being detected at the time of bisection, reflecting the difficulty in obtaining a thorough morphological evaluation. However, demi-embryos were cultured without zonae, whereas the control embryos were cultured with an intact zona pellucida. After 48 h of culture *in vitro*, nonbisected pig embryos in which the zona pellucida had been removed by a short-term pronase treatment contained more cells than did embryos cultured with an intact zona pellucida (Niemann *et al.*, 1983b). It is assumed that the zona pellucida can play an important role in the regulation of cell division and that, in the early absence of the zona pellucida, blastomere multiplication is enhanced. However, it is possible that the zona pellucida-free demi-embryos can fuse in the uterus to form viable chimaeras. Results from the analysis of the blood genetic markers suggest that this could occur in a small percentage of the piglets going to term.

In the study reported here, the percentage of transferable pairs was higher after bisection of blastocysts than that in morulae. In cattle, bisected blastocysts resulted in higher pregnancy rates than in morulae (Williams *et al.*, 1984; McEvoy and Sreenan, 1990). In small ruminants, expanded blastocysts and even hatched blastocysts yielded better pregnancy rates than did earlier stage embryos (Tsunoda *et al.*, 1985; Chesné *et al.*, 1987; Shelton, 1992). No such observations have been made in pigs, although embryos from day 4–7 were successfully bisected (Nagashima *et al.*, 1988, 1989; Ash *et al.*, 1989) and in our experiment no separate transfer of demi-embryos derived from either blastocysts or morulae could be done. Apart from the developmental stage of the parent embryos, we identified several other factors that had a significant effect on the percentage of transferable pairs of demi-embryos, such as quality of the parent embryos, duration of the culture *in vitro* before bisection and the individual donor (Niemann and Reichelt, 1993).

The transfer of 14–16 pig demi-embryos per recipient yielded only low (< 40%) pregnancy rates (Nagashima *et al.*, 1988, 1989; Ash *et al.*, 1989). These low pregnancy rates could have been due to a higher incidence of early embryonic mortality which regularly reaches 35% until day 25 of pregnancy (see Pope *et al.*, 1990). In contrast, we transferred an average of 28 demi-embryos per recipient and together with the selection of viable demi-embryos after 24 h of culture for transfer, this could have led to the high pregnancy rates of approximately 80%, which compare favourably with the pregnancy rates obtained after transfer of intact embryos (Polge, 1982; Niemann, 1989). Whereas the pregnancy rate obtained in our investigation was similar to that after transfer of intact embryos or after mating, litter size was considerably reduced. A similar finding was observed after transfer of intact embryos cultured *in vitro* (Pope and Day, 1977; Davis and Day, 1978; Niemann, 1989). It is not known whether the culture *in vitro* or the reduction in numbers of cells contributed more to the higher embryonic losses, whereas a crowding effect can probably be ruled out (Bazer *et al.*, 1969; Pope *et al.*, 1972). Our results suggest that the insufficient developmental potential *in vivo* of some of the transferred demi-embryos is the main reason for the relatively small litter size. We cannot rule out the possibility that demi-embryos classified into category I had a higher development *in vivo* than did demi-embryos scored into category II. But most of the transferred demi-embryos were

classified into category I. The data obtained *in vitro* clearly show that only excellent graded demi-embryos (category I) possessed developmental characteristics *in vitro* close to non-bisected controls. A separate transfer of demi-embryos of the two morphological categories to different recipients could clarify this question, which is important for large scale production of twin pigs.

When comparing the developmental potential of micro-surgically bisected embryos from various mammals, it is found that survival and twinning rates with demi-embryos in multiparous species are significantly lower than in cattle, sheep or goats (50–70% survival, 40–45% twinning) (Gatica *et al.*, 1984; Willadsen and Godke, 1984; Takeda *et al.*, 1986; Chesné *et al.*, 1987; Leibo and Rall, 1987; Seike *et al.*, 1989). In contrast, survival of demi-embryos in mice and rabbits is usually impaired and twinning rates of 2–5% have been obtained (Gärtner and Baunack, 1981; Tsunoda and McLaren, 1983; Nagashima *et al.*, 1984; Ponzilius *et al.*, 1987; Yang and Foote, 1987, 1990). Previously, pig four-cell embryos were bisected, transferred to foreign zonae pellucidae, embedded in agar, cultured *in vivo* in the sheep oviduct up to the blastocyst stage and blood typing then revealed two sets of identical twins among the piglets born after transfer to the final recipients (Polge, 1985). Nagashima *et al.* (1988) identified monozygosity in three sets of twin piglets via analysis of nine blood groups and eight proteins. Ash *et al.* (1989) found two monozygotic pairs of twin piglets via analysis of an unknown number of proteins and through reciprocal skin transplantation. However, in the latter study intact embryos were transferred with the demi-embryos and a significant competition was observed. It is impossible to calculate the twinning rate since in both investigations accurate data on the number of transferred pairs of demi-embryos are not provided. The seven pairs of identical twin piglets obtained in our investigation developed out of 311 pairs of demi-embryos transferred to the farrowing recipients and correspond to a twinning rate of 2.3%, which is similar to values reported for mice (Gärtner and Baunack, 1981; Ponzilius *et al.*, 1987). However, the fact that analysis for monozygosity did not include the stillborn piglets or those that died shortly after birth (a total of 31) should be taken into account as there could have been additional pairs of monozygotic twins.

The analysis of important parameters in pig production revealed some significant differences between the experimental gilts (recipients) and the mated controls. Litter size was significantly reduced in those gilts that had received demi-embryos. Potentially, this could have been attributed to a reduced developmental capacity of the transferred demi-embryos. However, puberty was induced by hormones in gilts that were used as recipients and it was shown that several endocrinological parameters of gilts in which puberty is induced differ from those of mature gilts, and this finding could be related to the higher early embryonic mortality observed in gilts bred at an induced pubertal oestrus (Rampacek *et al.*, 1985; Kineman *et al.*, 1987; Archibong *et al.*, 1987). However, in a previous study in the same herd, transfer of nonbisected pig embryos after 8–10 h of culture *in vitro* to gilts stimulated with a similar protocol led to the same litter size, as observed in the experiment reported here. Postnatal development of the piglets was normal and no differences were found between gilts and sows (Niemann, 1989). In addition, embryos recovered from

stimulated prepubertal gilts were shown to be cytogenetically normal and capable of regular full term development (Dolch and Chrisman, 1981; Niemann, 1989). Prepubertal gilts can be synchronized easily, have a facilitated handling and were available for our study. As another control, gilts could have received nonbisected embryos but because of the limited number of gilts available and the extended experience with this procedure, the present experimental groups were chosen. It is known that early embryonic mortality due to crowding in the uterus occurs within the first 25 days of pregnancy (Dziuk, 1968; Pope *et al.*, 1972). Further investigations involving detailed analyses of the various sections of the uterine horns could be useful for elucidating potential reasons for the low litter size. The average lower birth weight also showed a larger standard deviation in the piglets from the experimental sows and the fact that these piglets were derived from small litters, which normally have a higher birth weight than piglets from larger litters, leads to the suggestion that at least some bisected embryos undergo impaired fetal development. The greater variation in birth weight could also have contributed to the higher losses of piglets derived from demi-embryos in the early *postpartum* period.

When comparing birth and weaning weight as well as daily weight gain between the two partners of a monozygotic pair, it is evident that some of the pairs displayed marked differences. In some pairs, the difference observed in birth weight was not apparent at weaning; in others it was observed consistently. Studies involving murine, bovine and human monozygotic twins have shown considerable variation between the two partners which was not obviously attributable to the environment (Gärtner and Baunack, 1981; Levine *et al.*, 1987; Gärtner *et al.*, 1991). These differences were attributed to intangible variation and our results suggest that this variation also occurs in pigs. These, in some cases, distinct differences between monozygotic twin partners could hamper the use of identical twins in some areas of biological research.

In addition to the usual methods of blood typing, DNA fingerprinting was used for the first time to identify monozygosity in pigs. DNA fingerprinting was used to investigate monozygotic fertilization in Large White and the prolific Meishan pigs and a higher incidence of monozygotic fetuses was found in the latter (Ashworth *et al.*, 1993). The accuracy of diagnosis of monozygosity depends on the number of genetic markers analysed, the degree of polymorphism and the gene frequencies. In some of the markers, the degree of polymorphism was very low and the gene frequencies were high. In our case it had to be taken into account that monozygosity was diagnosed among full siblings and that the animals could not be considered as genetically independent from each other. In humans, monozygosity among twin births can be diagnosed with an accuracy of 95–98% using traditional methods (Nylander, 1970). The confidence level of diagnosing monozygosity with procedures involving multiple gene loci can be calculated as the likelihood of identity (see Jeffreys *et al.*, 1985). This likelihood represents the probability that two individuals are by chance identical at all gene loci. On the basis of the assumption that the population was in the Hardy Weinberg equilibrium and there was no linkage disequilibrium and using the 25 traditional pig blood genetic markers, the calculated probability for each animal of its genotype occurring twice by

chance ranged from 3.8×10^{-4} to 2.4×10^{-10} . Under the same assumption, for DNA fingerprinting using only the best visible bands ($n=18$) involving the banding pattern of 22 animals, this probability was calculated to be less than 1×10^{-7} . However, this is based on a conservative calculation model taking into account the possibility that different bands may not separate independently and involved the bands in the upper region only. The combination of blood typing plus DNA fingerprinting increased the probability to a range from 3.8×10^{-11} to 2.4×10^{-17} . Future efforts will be directed towards simplifying the determination of monozygosity, for example by applying DNA fingerprinting alone. The likelihood of identity calculated in our experiment for DNA fingerprinting would suggest that this is feasible.

In summary, results from our investigation showed that, in contrast to previous suggestions (Robl and First, 1985), pig morulae and blastocysts can be bisected relatively easily and yield large numbers of viable demi-embryos. When transferring a sufficient number of demi-embryos per recipient, high pregnancy rates can be obtained, although litter size is reduced. Among the piglets born, approximately 2% identical twins can be positively identified with sufficient security by blood typing and DNA fingerprinting. Monozygotic twin piglets can serve as excellent models in various areas of biological research. The monozygotic twin piglets identified in this study underwent reciprocal skin and pancreas transplantations without showing any type of rejection of the respective graft. Further investigations are necessary to identify factors relevant to the developmental potential of porcine demi-embryos.

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