NON-SURGICAL EMBRYO TRANSFER IN PIGS

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NON-SURGICAL EMBRYO TRANSFER IN PIGS

Wouter Hazeleger

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 29 juni 1999
des namiddags te half twee in de Aula

VOORWOORD

Eindelijk een proefschrift, na ruim 10 jaar ET onderzoek en 25 jaar bij de universiteit werkzaam geweest te zijn. Aan dit langdurige proces hebben veel mensen een bijdrage geleverd, die ik hierbij wil bedanken. Allereerst Auke Osinga, bedankt voor de beslissing om me aan te nemen. Jij hebt de basis gelegd, onder meer door me "op te zadelen" met het min of meer zelfstandig uitvoeren van diverse experimenten. Nadat je was opgevolgd door Tette van der Lende, ontstond het idee van niet-chirurgische embryo-transplantatie bij varkens. Tette, heel hartelijk bedankt voor je stimulerende houding en initiatieven om dit werk in de vorm van een promotieonderzoek uit te gaan voeren. Ondanks je vertrek en wisselingen in banen ben je altijd een onmisbare steun en toeverlaat voor me geweest. Jos, promotor, waarschijnlijk heb je fronsend de voorstellen van Tette aangehoord, maar ik ben je bijzonder dankbaar dat je me de ruimte hebt gegeven om dit werk te doen. Hoewel je meer als indirecte begeleider functioneerde, was je positieve benadering en belangstelling voor dit werk van wezenlijk belang om de moed erin te houden. Bas, co-promotor, bij je aanstelling opgescheept met een analist die wilde promoveren, bedankt dat je me de kans gaf om dit werk voort te zetten. Je enthousiasme en stimulerende pep-talks zijn onmisbaar voor me geweest en zijn dat ook nu nog steeds. Niet alleen als co-promotor en als leider van de reproductie groep, maar vooral als collega (en kamergenoot bij congressen) waardeer ik je bijzonder. Nicoline, tijdens je eigen promotieonderzoek geplaagd door een medewerker die embryo-transplantatie minstens zo belangrijk vond als scannen. Bedankt voor al je hulp en positief-kritische kanttekeningen en opmerkingen bij de concept artikelen. Je bent altijd een fantastische collega geweest. Laten we dit zo houden. Emmy, als laatste bij dit werk betrokken geraakt. Wat een enthousiasme, alsof het je eigen onderzoek was. Bedankt voor al die nachtelijke uurtjes, weekend-werk, slachthuisbezoeken in de vroege ochtend en al dat soort zaken. Nicoline en Emmy, ik ben blij en voel me vereerd dat jullie me ook tijdens de laatste uurtjes van dit promotieonderzoek als paranimfen willen bijstaan.

Ook andere collega's waren onmisbaar. Voor verschillende nevenactiviteiten heb ik me wel eens wat minder in kunnen zetten, wat zonder (voor mij hoorbare) problemen door mijn collega's geaccepteerd werd. Mike, mijn eeuwige (25 jaar) collega, praatpaal, kamergenoot, enz., bedankt en hopelijk nog vele jaren zo. Frits, Ger en PC-Henk, bedankt voor jullie hulpvaardige instelling. Alle andere (oud-) collega's (OBP + WP + AIO's), jullie aanwezigheid, discussies en collegiale instelling tijdens koffie en bij andere gelegenheden, zijn van groot belang geweest om goed te functioneren.

Een werkelijk onmisbare groep waren de studenten en stagiaires. Al die deelonderzoeken, vaak eindigend in scripties met (frustrerende?) filosofieën over waarom het allemaal mis ging en hoe het beter zou kunnen, zijn van wezenlijk belang geweest om

STELLINGEN:

1. Op zowel Dag 4 als Dag 5 na ovulatie is de op dat moment gerealiseerde morfologische ontwikkeling van varkensembryo's bepalend voor de overlevingskansen na niet-chirurgische embryo transplantatie.
   Dit proefschrift.

2. De optimale voorwaarden voor succesvolle, niet-chirurgische embryo-transplantaties bij varkens verschillen van de optimale voorwaarden voor succesvolle, chirurgische transplantaties bij varkens.
   Polge C, 1982. In: Cole DJA, Foxcroft GR, (eds), Control of Pig Reproduction. London: Butterworth Scientific pp 279-291.
   Dit proefschrift.

3. Een juiste selectie van donorzeugen is belangrijk voor een goede embryo-opbrengst.
   Dit proefschrift.

4. Embryo-transplantatie bij varkens biedt momenteel de beste garanties voor het transporteren van ziektevrij genetisch materiaal.

5. De ontwikkelde niet-invasieve techniek om via de cervix de uterus van zeugen te bereiken, biedt nieuwe mogelijkheden om effecten van (beer)stimuli op uteruscontracties van zeugen te onderzoeken.

6. Indien de bij muizen aangetoonde negatieve interactie van cellulaire en positieve interactie van humorale immunologische reacties met embryonale ontwikkeling en overleving ook bij landbouwhuisdieren en de mens bestaat, dient het vaccinatie beleid aangepast worden aan de reproductieve status van het vrouwelijk dier of mens.

7. Het gebruik van roestvrijstalen instrumenten in de medische sector reflecteert een weinig soepele benadering van de anatomie van mens en dier.

8. De herkenbaarheid van een organisatie vermindert door frequent de naam te veranderen.

9. Bij de discussie over de welzijnsproblematiek in de veehouderij wordt te weinig aandacht besteed aan de interactie tussen mens en dier.

10. De uitdrukking "A rolling stone gathers no moss" is achterhaald door "The Rolling Stones".

W. Hazeleger
Non-surgical embryo transfer in pigs
Wageningen, 29 juni 1999

erachter te komen hoe het allemaal wel moest. Daarom Anja, Annie, Bert, Carla, Claudia, Erik, Frank, Gerjan, Gerrianne, Hans, Frank, Ina, Ivar, Jos, José, Michiel, Patrick, Paulien, Sam, Tjalling, allemaal bijzonder hartelijk bedankt voor jullie inzet.

Verder zijn vele anderen onmisbaar geweest om dit werk goed te kunnen doen. Allereerst de collega's op de proefaccommodatie, Jan, Ries, André, Ben, Sjaak en alle andere mensen, die altijd wel een oplossing wisten of maakten voor de (ruimtelijke) problemen van de vele dieren die in het onderzoek gebruikt werden. Verder natuurlijk de leden van de voormalige, voor mij inspirerende, "Werkgroep Vroege Dracht", waarvan met name Frans Helmond en Jan van der Meulen bij de operaties belangrijk zijn geweest. Natuurlijk Slachthuis Hallers en Slachthuis Veenendaal waar Ko van Ginkel en zijn medewerkers bereid waren om zelfs tijdens het weekend of feestdagen te helpen. Financiële ondersteuning van het bedrijfsleven is onmisbaar gebleken. Dalland, Euribrid, Dumeco Breeding, Stamboek, Maatschap van Varkens KI verenigingen en PVE worden bij deze nogmaals hartelijk bedankt voor hun bijdrage. Wim Cöp, Henk Grooten, Egbert Knol, Jan Merks, Bart Molenaar en Freerk Wind, een extra woord van dank voor jullie morele steun. Dankzij jullie is er een nieuwe generatie mensen met varkens-ET aan de slag.

Helaas ben ik er vrij zeker van dat ik iemand met een zeer wezenlijke bijdrage in dit werk nog niet genoemd heb. Na inlevering van dit werk, of bij een ontmoeting loop ik beslist een keer met een rood hoofd rond; mijn excuses. Allen die een directe of indirecte bijdrage geleverd hebben aan dit werk, nogmaals hartelijk bedankt.

Het belangrijkste van alles, mijn vrouw en kinderen, het thuisfront. Al 25 jaar in goede banen geleid door Joke. Met een man of pa die soms op de meest vreemde tijdstippen werkt, vaak te laat thuiskomt, zo af en toe wat afwezig in of rond het huis ronddoolt of met een hoofdtelefoon op achter de PC zit, wat moeten ze er mee. Behalve bij te laat thuiskomen is er vrijwel nooit (in mijn bijzijn) over gemopperd. Als ik weer thuis was, werd ik gelukkig onmiddellijk geconfronteerd met de realiteit van het gezinsleven. Hierdoor is een werkverslaving voorkomen en is het werk daardoor een beperkt, hoewel niet onbelangrijk, onderdeel van mijn leven gebleven. Joke, Gerard (omslag!), Corine en Willy, bedankt. Jullie zijn mijn grootste trots. Aan jullie draag ik dit werk dan ook graag op.

Wouter

Omslag: Gerard Hazeleger 1999

ISBN: 90-5808-072-2

Hazeleger, W., 1998. Non-surgical embryo transfer in pigs.

Embryo transfer in pigs has been performed surgically for a long time. Non-surgical procedures were considered to be impossible to perform due to the complex nature of the genital tract and the location of the embryos in the top of the uterine horns. However, a less invasive and more natural, non-surgical, procedure of embryo transfer could be a valuable tool for research (to study embryo survival and embryo-uterus interactions) and practical applications (export, prevention of disease transmission and prevention of long distance transport of animals).

Therefore, the aim of this study was to develop non-surgical procedures for the collection and transfer of porcine embryos which can be used for both practical and research purposes. The development of these procedures was expected to give insight in factors which are important for embryonic survival.

Non-surgical (trans-cervical) collection of embryos appeared to be possible from sows with surgically resectioned uterine horns. On average 8 normal developing embryos could be collected from these sows. After superovulation induction the average yield was increased to 18 embryos, with a large variation between sows.

Non-surgical (trans-cervical) transfer of embryos to non-sedated recipients appeared to be possible. A pregnancy rate of 60% and a litter size of 11 foetuses on Day 35 of pregnancy was realised. These results appeared to be dependent on the asynchrony between donor and recipient sows and on the development of the transferred embryos. At Day 4 or Day 5, the best developed embryos resulted in the highest pregnancy rates. No relations between characteristics of recipients, like age or previous litter size, with pregnancy rates were found. Also no relations between scores of the procedure of embryo transfer (like resistance in the cervix, duration of the procedure, etc.) and pregnancy results were found. Therefore factors affecting embryonic development and their survival after non-surgical transfer need further attention.

PhD Thesis, Wageningen University, Department of Animal Sciences (Animal Health and Reproduction Group), Wageningen Institute of Animal Sciences (WIAS), P.O.Box 338, 6700 AH Wageningen, The Netherlands.

Parts of this study have been financially supported by: Dalland bv, Dumeco Breeding, Dutch Association of AI Centres for Pigs, Dutch Herdbook bv, Euribrid bv and Product Board for Livestock, Meat and Eggs.

Contents:

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Embryo transfer has always been a valuable tool for research purposes. With the aid of (surgical) embryo transfer procedures important knowledge on the relation between embryonic development and embryonic survival, as well as embryo-uterus interactions was obtained (e.g. Pope et al. 1982). For practical applications embryo transfer has been used only to a limited extent (e.g. Cameron et al. 1990). This was due to limited applicability of surgical embryo transfer at the farm level. Furthermore, in pig breeding the need for extra offspring is low, due to the high fecundity of pigs. However, for certain aspects in pig breeding, like export of genetic material (prevention of long distance shipment of animals) and disease control, as well as for future applications of new embryo technologies, it is a valuable tool. This has increased the motivation to use embryo transfer in pig breeding (Martin 1983, Polge 1985).

In pigs, embryo collection and transfer have been performed surgically since 1962 (Hancock and Hovell). On average a pregnancy rate of 60% and a litter size of 6.5 piglets has been realised (Brüssow and König 1988). The main disadvantages are that the animals are subjected to invasive (i.e. surgical) procedures and that these surgical procedures need special facilities like a surgery room and adequate equipment for anaesthesia. The recently developed endoscopic procedure for embryo transfer, with realised pregnancy rates of 90% (Besenfelder et al. 1998), is considered as minimally invasive, but it remains a surgical procedure with its specific disadvantages.

Considering the disadvantages of surgical procedures, non-surgical procedures are undoubtedly preferable. Such procedures have the advantage that the animals are not subjected to surgery and that they can be performed on-farm, without need for special facilities. The successful application in cattle of non-surgical embryo collection and transfer as well as related new reproduction techniques (e.g. embryo freezing, ultrasound-guided trans-vaginal ovum pick-up, in vitro embryo production) have stimulated the demand to develop similar techniques for use in pigs. After the first unsuccessful attempt of Polge and Day (1968) to transfer porcine embryos non-surgically, non-surgical embryo transfer has been considered as an impossible technique for a very long time (Polge 1982). The major obstacles for a non-surgical technique were the complex nature of the genital tract with its constricted cervical canal and long, coiled uterine horns as well as the location of the embryos in the top of the uterine horns (i.e. close to the utero-tubal junction) during early pregnancy.

Aim of this research

The aim of this research was to develop non-surgical procedures for the collection and transfer of porcine embryos which can be used for both practical and research purposes. The development of these non-surgical procedures was expected to give insight in factors which are important for embryonic survival.

The development of non-surgical procedures for embryo collection and embryo transfer was preceded by the development of a non-surgical (i.e. trans-cervical) procedure to obtain access to the lumen of the uterine body. After many trials using slaughterhouse material, an instrument and procedure was developed which enabled the introduction of a catheter into the uterine lumen of non-sedated primi- and multiparous sows (Hazeleger and Van der Lende 1987).

Embryo collection

During early pregnancy, the embryos are located in the top of the uterine horns, near the utero-tubal junction. Each horn is 1 to 2 m long and is positioned in the abdominal cavity in a coiled and twisted way. Therefore, transcervical flushing of the top of the horns is impossible under normal physiological conditions. A solution for this problem is described in Chapter 1. In this chapter a surgical procedure is described which can be used to permanently shorten the uterine horns by detachment of a large central part and connecting the top and the base of each uterine horn. In this way, the shortened uterine horns can be flushed for embryo collection. In Chapter 2 the results of the non-surgical embryo collection procedure, using sows with surgically shortened uterine horns, are described and evaluated. Although it was possible to collect embryos routinely in this way, the yield was always highly variable.

Embryo transfer

Subsequent research focussed on the development of a procedure for transcervical transfer of embryos. All experiments for this purpose were performed with embryos collected from donor sows which were slaughtered at Day 4 or 5 after ovulation. In this way embryos can be relatively easily obtained and high yields are possible. The transcervical transfer procedure allows deposition of the embryos in the lumen of the uterine body or caudal end of the uterine horns. Normally, such embryos are located near the utero-tubal junction, i.e. in the upper part of the uterine horns. Although no clear evidence is available that the uterine environment in the uterine body differs from that in the top of the horns, some differences might be assumed. It can not be excluded that these differences affect embryonic survival. In

one of the first experiments it had been shown that embryos will develop after the non-surgical transfer procedure into the lumen of the uterine body for at least two days (Hazeleger et al. 1992).

In Chapter 3 the results of an experiment, in which Day 4 embryos were transferred, are presented. It appeared that only the best developed Day 4 embryos resulted in pregnancies. Subsequent research therefore focussed on Day 5 embryos, which might possibly be more competent to survive the uterine environment in or near the uterine body. In Chapter 4 the requirements for synchrony between donors and recipients was tested with Day 5 embryos.

The next step was the further improvement and refinement of the transfer procedure and the transfer instrument. The latter resulted in a patent application (Hazeleger 1997). This stage of the research also aimed at finding descriptive parameters which could indicate the quality of and useful selection criteria for the embryos at the moment of transfer. In a pilot experiment it appeared that of the Day 5 embryos (120 h after ovulation), expanded blastocysts had the best survival chances after transfer.

In Chapter 5 effects of superovulation (low and high dose of eCG) on number of transferable embryos, embryonic development and subsequent survival after transfer of expanded blastocysts were studied. In Chapter 6 factors other than embryonic development, which might contribute to the success of the transfer procedure, were studied. Therefore data of a pilot experiment and the data from Chapter 5 were combined for analysis. In both experiments only transfers with expanded blastocysts were analysed to find characteristics of the transfer procedure (like the resistance felt at introduction of the instrument, duration of the transfer procedure, etc.) and characteristics of the recipients (like parity, weight, previous litter size, etc.) which could contribute to the success of the transfers. Also relations between characteristics of the donors and the number of transferable embryos were studied.

In the General Discussion, the procedures and results of embryo collection and embryo transfer, were compared with and related to the procedures and results of other groups working on non-surgical embryo transfer in the pig (Kobayashi et al. 1989, Reichenbach et al. 1993, Galvin et al. 1994, Li et al. 1996, Yonemura et al. 1996). Furthermore, the prospects for application of non-surgical embryo transfer in the pig breeding industry are discussed.

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Chapter 1

A METHOD FOR TRANSCERVICAL EMBRYO COLLECTION IN THE PIG

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Published in:
Theriogenology 32 (1989) 727-734

A METHOD FOR TRANSCERVICAL EMBRYO COLLECTION IN THE PIG

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ABSTRACT

Five cyclic primiparous sows were used to test a surgical procedure for in vivo transcervical collection of pig embryos. The procedure consisted of shortening the uterine horns. After surgery, all sows returned to oestrus and embryos were recovered following artificial insemination. Transcervical uterine flushing was carried out in four sows. On average 3.6 ± 1.5 (mean ± SD) embryos were recovered from the five sows. The results indicate that it is possible to recover embryos transcervically from sows with a resectioned uterus.

Key words: embryo collection, transcervical, uterine resection, pig

INTRODUCTION

The techniques used to collect embryos from sows for research and/or embryo transfer have changed little since the early 1960s (Hancock and Hovel 1962, Dziuk et al. 1964, Vincent et al. 1964, Day 1979, Polge 1982, Morgan et al. 1987), and no successful method for collecting embryos repeatedly non-surgically from the same sow has been reported. The major problem that hinders non-surgical collection of embryos is the anatomy of the cervix and uterus. The cervical folds make the transcervical introduction of a catheter into the uterus difficult. Moreover, the length and coiled nature of the uterine horns prevent the safe flushing of the embryos that are located at the top of the uterine horns at Days 3 to 7 after insemination.

A reliable non-surgical method for porcine embryo collection and transfer would benefit the pig industry in terms of production and disease control. Transcervical embryo collection might be accomplished by shortening the uterine horns surgically and collecting embryos using the transcervical uterine catheterisation procedure of Hazeleger and Van der Lende (1987).

The aim of this study was to investigate whether it is possible to collect embryos transcervically from sows with resectioned uterine horns on Day 5 or 6 after insemination.

MATERIALS AND METHODS

For this study, three Great York x Dutch Landrace and two Pig Improvement Company (PIC; Oxford, England) primiparous sows, weighing 120 to 210 kg, with regular inter-oestrus intervals were used. Uterine horn resectioning was performed between Days 10 and 15 of the oestrus cycle to remove a segment of each horn. Using sterile procedures, the uterine horns were exposed by lateral laparotomy under general anaesthesia as previously described (Van der Meulen et al. 1988). The resectioning was begun by separating the last portion (5 to 10 cm) of the top of each horn from the rest of the horn up to the mesometrium. This procedure was repeated at the base of the horn near the bifurcation (Figure 1A). The top and base of each horn were connected with a continuous suture (Figure 1B). The remaining segment of each horn (the excluded horn) was left in situ, after closing the open ends with a purse-string suture. The last four sows were infused with an antibiotic into the uterine lumen of the resectioned and the excluded horns of the uterus.

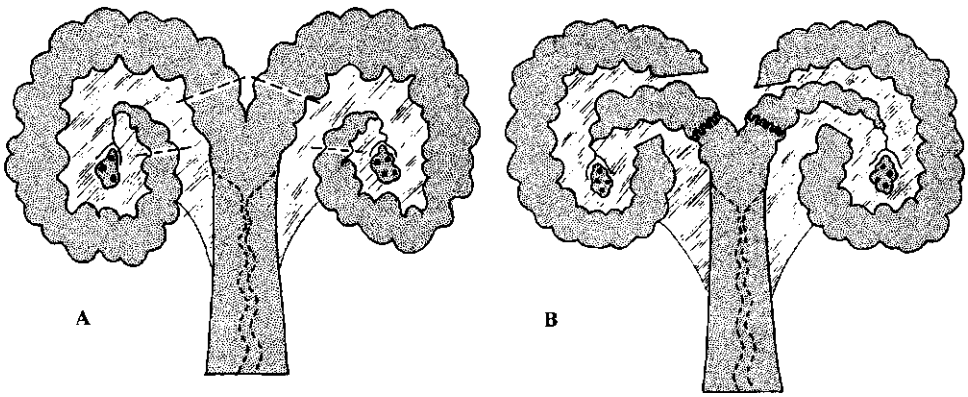

Figure 1. Schematic presentation of the uterine resection procedure.
A: The dotted lines indicate approximate points of separation of the middle, top and base portions of the horns.
B: Schematic view of the resulting resectioned uterus.

On the first day of the second oestrus following surgery, the sows were artificially inseminated with 80 mL of diluted fresh semen. The first sow, being a test case for the surgical procedure, was slaughtered on Day 6 after insemination to examine the results of surgery and to determine the number of embryos present. The uterus was obtained immediately after slaughter and stored on ice until inspection and flushing at our laboratory. The uteri of the four other sows were transcervically flushed on Day 5 or 6 after insemination. Two of these sows were slaughtered 2 h after flushing to examine the results of surgery and efficiency of the flushing procedure.

The embryo collection in vivo was carried out by transcervical catheterisation of the uterus, as described by Hazeleger and Van der Lende (1987). The unsedated sow was placed in a metabolic cage for optimal presentation. During handling, feed was available ad libitum. A solid polyvinylchloride (PVC) bar (length 70 cm; diameter 3 mm), of which the last centimetre made an angle of 90° with the rest of the bar, was inserted into the uterus by moving the bar through the cervix with gentle rotation and pressure (Figure 2A). Once the tip of the bar was passed through the cervix (at which resistance ceased), a straight solid PVC bar (length 1 m; diameter 3 mm) was carefully inserted parallel to the first bar (Figure 2B). After removal of the first bar a polyethylene (PE) tube (length 70 cm; inner diameter 5 mm; outer diameter 7 mm) was passed over the straight bar (Figure 2C). When the tube was correctly placed through the cervix, the straight bar was removed (Figure 2D). Through the tube a PE catheter (inner diameter 3.5 mm; outer diameter 4.5 mm) with several lateral holes near a blunt top to prevent damage of the endometrium, was inserted into the uterus (Figure 2E). Flushing was carried out by infusing and retrieving 10 or 20 mL of Dulbecco's PBS several times through the catheter.

After slaughter embryos were collected by flushing the resectioned uterine horns from the corpus uteri to the utero-tubal junction with 2 x 20 mL of PBS.

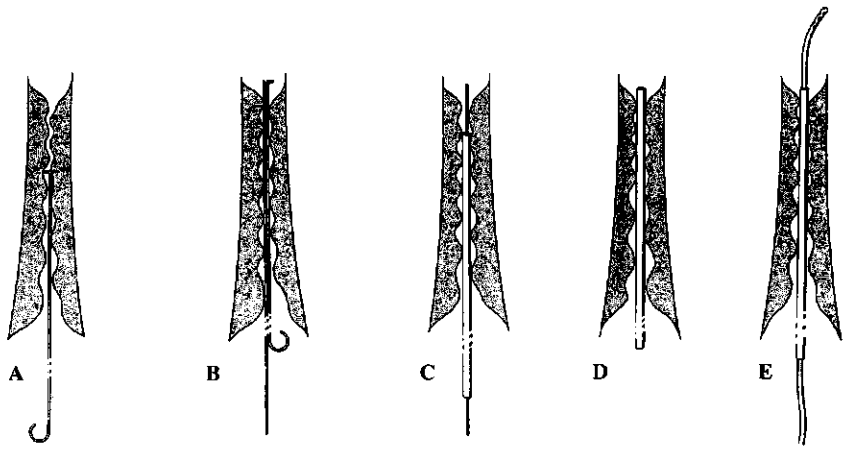

Figure 2. Schematic presentation of the transcervical uterine catheterisation.
A: The initial bar with hook in the cervix.
B: The straight bar along the initial bar.
C: The hooked bar removed and the tube partially shifted over the straight bar.
D: The tube correctly placed through the cervix.
E: A catheter shifted through the tube into the uterus.

RESULTS

The intervals from surgery to oestrus and from oestrus to flushing, inter-oestrus intervals, numbers of corpora lutea, recoveries of embryos and volumes of flushing medium (in vivo flushing) are presented below (Table 1).

The first sow recovered slowly following a long operation and returned to oestrus 11 d later than expected. At slaughter, 9 of the 12 corpora lutea on the left ovary were enclosed by adhesions of the fimbriae. The fimbriae and right ovary with four normal corpora lutea had no adhesions. The left excluded uterine horn contained a purulent fluid, presumably due to endometritis. The right excluded horn contained some haemorrhagic fluid. The resectioned horns had a normal appearance. After slaughter, two embryos were flushed from each resectioned horn.

The other four sows showed regular oestrus intervals following surgery. At examination of the uteri of Sows 2 and 3, which were slaughtered after flushing, some mild adhesions of the fimbriae on one ovary were detected, and in Sow 2 the adhesions enclosed one corpus luteum. The excluded horns contained some haemorrhagic fluid. No other adhesions or disturbances due to the surgical procedure were detected macroscopically. The results of a uterine resection are shown below (Figure 3).

Flushing of the uterus of Sow 4 gave problems due to leakage of flushing fluid. Only one embryo was recovered. The flushing procedures in the other sows gave no problems due to leakage and resulted in recoveries of three to four embryos. On average 3.6 ± 1.5 (mean ± SD) embryos were recovered from the five sows.

Table 1. Oestrus parameters and embryo recovery rates following uterine resection in the sow.

Sows	1	2	3	4	5
Days between					
- surgery and preceding oestrus	15	13	15	14	10
- surgery and 1st oestrus p.s.	17	7	9	8	10
- 1st and 2nd oestrus p.s.	18	18	21	20	20
- 2nd oestrus p.s. and flushing	6	6	6	6	5
- 2nd and 3rd oestrus p.s.	sl.	sl.	sl.	21	21
Number of CL	16	16	15	n. sl.	n.sl.
Number of recovered embryos					
- after flushing	n.f.	3	4	1	4
- after slaughter	4	2	0	n. sl.	n.sl.
Volume (mL) of in vivo flushing medium					
- infused	n.f.	60	60	150	380
- retrieved	n.f.	50	50	110	360

p.s. = post surgery.

sl. = slaughtered for embryo recovery.

n.sl.= not slaughtered.

n.f. = not flushed in vivo.

Figure 3. The resectioned uterus after slaughter. The mesometrium of the excluded horns was partially dissected to improve exposure of the resectioned horns.

DISCUSSION

These results show that sows with resectioned uterine horns return to oestrus after surgery and that it is possible to recover embryos transcervically after artificial insemination.

The results for the first sow (ovarian adhesions, fluid in excluded horn, extended first oestrus) indicate suboptimal operating conditions. The results for the other sows were better, probably due to a shorter duration of surgery and intrauterine antibiotic treatment. There remained, however, some mild adhesions of fimbriae on some ovaries, indicating that more careful handling of the ovaries is necessary. Whether these adhesions caused the low number of embryos collected needs to be investigated. The low number of embryos is not due to the number of ovulations, as judged by the number of corpora lutea (Table 1).

The resectioning of the uterus has virtually no effect on the position of the uterine horns in the abdomen, since in intact sows the top and the base of the horns are in close proximity. Therefore these parts are easily connected without severe disturbance of the normal position of the horns in the abdomen. The resulting resectioned uterine horns are able

to contain fertilised ova as was demonstrated in this experiment. Several authors have shown that severe restriction of space in the uterine horns does not reduce the number of embryos during early pregnancy (Dziuk 1968, Fenton et al. 1970, Webel and Dziuk 1974). The reduced space after the resectioning of the uterine horns is therefore not expected to limit the number of embryos during early pregnancy.

The low recovery rate of embryos after slaughter in Sows 2 and 3 may have been due to loss of the remaining embryos with the residual flushing fluid. Fluid may be lost during the period between flushing in vivo and flushing after slaughter. Whether the low recovery rates in vivo are due only to the flushing procedure used or are caused by the surgical resectioning of the uterine horns needs to be investigated. The flushing procedure does not allow for the precise guidance of the catheter into the horn of choice. The procedure might be improved by introducing the instruments under rectal guidance and by using a Foley catheter with an inflatable cuff to flush the resectioned horns separately. This may prevent the leakage of flushing medium and increase the recovery rate of embryos.

An attempt (Altenhof et al. 1982) to collect embryos non-surgically was based on a hormonal treatment of sows for the expulsion of the embryos. The results of these authors indicated movement of the embryos in the direction of the cervix, but they were not successful in their efforts to collect embryos non-surgically. The method of collecting embryos presented in our study is comparable to conventional non-surgical embryo collection procedures used in cows (Peters and Ball 1987). Transcervical uterine catheterization in pigs has also been described (Polge and Day 1968) for embryo transplantation experiments. Evidence was provided that pregnancy can be established in this way, despite problems with the catheterization procedure used. Our method of uterine catheterization causes no severe stimulation or damage of the genital tract; however, the use of primiparous or pluriparous sows is important because gilts have a more constricted cervix.

Our method for uterine catheterization may also be of value for embryo transplantation, because no surgical procedures are needed. A few studies indicate that early embryos do not need to be located in the top of the uterine horns for normal development. Stein-Stefani and Holtz (1987) showed that Day-5 embryos are able to develop normally after transplantation to the middle region of the uterine horns, and according to Polge (1977), four-cell embryos develop normally after transplantation to the cervical end of the uterus. This indicates that successful non-surgical transfer of embryos into the cervical part of the uterus is possible.

In other polytocous species, an analogous procedure may be used for non-surgical embryo collection and transfer, because these species present comparable anatomical problems (Hurlbut et al. 1988; Goodrowe et al. 1988). Moreover, non-surgical embryo transfer procedures may also be useful for enhancing reproduction.

In conclusion, after surgical resectioning of sow uterine horns, Day-5 or Day-6 embryos can be collected by means of transcervical uterine catheterization. This procedure offers new possibilities for non-surgical embryo collection and transfer which would benefit the pig industry.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the animal care staff for their technical assistance and care of the sows. This work was carried out as a project of the Research Group on Early Pregnancy at the Agricultural University, Wageningen, The Netherlands.

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Chapter 2

TRANSCERVICAL EMBRYO COLLECTION AND REPRODUCTIVE PERFORMANCE OF SOWS WITH RESECTIONED UTERI.

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Published in:
Reproduction in Domestic Animals 29 (1994) 38-45
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TRANSCERVICAL EMBRYO COLLECTION AND REPRODUCTIVE PERFORMANCE OF SOWS WITH RESECTIONED UTERI.

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ABSTRACT

Three experiments were performed. In Exp. 1 the reproductive performance of sows with resectioned uteri and the embryonic recovery after repeated transcervical collection and after slaughter was determined. In Exp. 2 the embryonic development in sows with resectioned uteri was compared with the embryonic development in intact sows. In Exp. 3 the embryonic recovery (after transcervical collection) and development were studied in three consecutive oestrous cycles with superovulation induction in Cycle 2.

After uterine resectioning in 16 sows, three sows were culled, two due to endometritis and one due to repeated cervical passage problems. The 13 other modified sows remained cyclic and eggs/embryos could be recovered repeatedly by transcervical flushing on Day 5 or 6 after insemination.

In Exp. 1 the length of the oestrous cycle of six modified sows increased from 20.2±1.2 to 21.2±1.3 (n=18; $P<0.05$) after transcervical embryo collection. Transcervical recovery was 6.2±4.2 eggs/embryos per collection (n=11). After slaughter the average number of recovered eggs/embryos was 12.0±6.0 (n=6). No major adhesions or other disturbances of the genital tract were detected after slaughter and the number of CL was normal (19.7±2.5).

In Exp. 2 the transcervical recovery was 6.3±3.4 (n=7) eggs/embryos. An average of 85.0±19.1% (n=4) and 80.5±39.6% (n=6) morula and blastocysts were collected from modified sows and intact control sows, respectively ($P>0.05$). The embryonic cell cycles were on average 4.0±0.9 (n=4) and 4.2±1.5 (n=6) per sow for modified sows and intact control sows, respectively ($P>0.05$).

In Exp. 3 the transcervical recovery was 8.1±5.5, 18.4±25.0 and 8.0±6.3 eggs/embryos in Cycle 1, 2 (superovulated) and 3, respectively ($P>0.05$). The % retarded embryos (2-8 cells) was 6.7±14.9, 24.7±20.2 and 1.9±3.2 ($P<0.01$) and the % morula and blastocysts was 89.0±13.7, 59.7±26.3 and 89.8±13.1 ($P>0.05$) in Cycle 1, 2 and 3, respectively. The average embryonic cell cycles were 4.4±0.7, 3.3±0.6 and 3.5±0.4 in Cycle 1, 2 and 3, respectively ($P<0.01$).

It is concluded that repeated transcervical embryo collection is possible in sows with resectioned uteri, without causing major reproductive disorders. Superovulation can increase the recovery, but the results are very variable.

Key words: embryo collection, transcervical flushing, uterine resectioning, pig

INTRODUCTION

New perspectives for embryo transfer in pigs occurred recently with the development of non-surgical embryo collection and transfer techniques (Kobayashi et al. 1989, Hazeleger et al. 1989, Sims and First, 1987, Killian et al. 1989, Hazeleger et al. 1992, Reichenbach et al. 1992). For optimal non-surgical embryo transfer procedures it is attractive to be able to collect embryos without need of surgery or slaughter of the donors. Therefore, donor sows with shortened uterine horns and a technique to collect the embryos transcervically are needed as described by Kobayashi et al. (1989) and Hazeleger et al. (1989).

Research of Kobayashi et al. (1989) showed that the cyclicity of sows with shortened uterine horns is disturbed due to persistent corpora lutea, probably caused by the partial hysterectomy performed. The resectioning procedure used by Hazeleger et al. (1989) might overcome these problems because all uterine tissue remains in the sow.

In the previous studies (Kobayashi et al. 1989, Hazeleger et al, 1989) the recovery remained rather low, while for practical application it is necessary to collect large numbers of embryos, which might be achieved by superovulation induction.

In this study three experiments were performed. The first experiment was conducted to evaluate the reproductive characteristics (cyclicity, number of CL and number and development of Day 5 embryos) of sows with surgically shortened uterine horns using the resectioning procedure of Hazeleger et al. (1989). The effect of transcervical flushing on cyclicity was also studied. In the second experiment the recovery and development of transcervically collected embryos was compared with the recovery and development of embryos collected by slaughter from intact sows. In the third experiment the effect of superovulation induction on the recovery and development of transcervically collected embryos was evaluated.

MATERIALS AND METHODS

Animals

In 16 crossbred (York x Landrace) sows uterine resectioning was performed between Days 10 and 15 of the oestrous cycle. As described and diagrammed by Hazeleger et al. (1989), uterine horns were shortened by connecting the top (5-10 cm) of the uterine horns with the base near the bifurcation, with minimal disturbance of the vascular system. The remaining segments (excluded horns) were closed and left in situ. The transcervical embryo

collections started after a recovery period of at least 25 days (second oestrus after resectioning). In Exp. 2 intact sows (York x Landrace; n=6) were slaughtered to collect control embryos.

Experiment 1

Six sows with resectioned uteri were tested for oestrus once daily in the morning with a vasectomised teasing boar. During a period of 9-11 oestrous cycles after uterine resectioning, the sows were inseminated in three non-consecutive oestrous cycles with an 80 mL commercial dose of fresh diluted semen (3×10^9 sperm cells) on the afternoon of the first day of oestrus.

On Day 5 or 6 after insemination animals were transcervically flushed for egg/embryo collection. Seven of these flushings were used to develop a modified transcervical flushing procedure (Fig. 1) based on the procedure described and diagrammed by Hazeleger et al. (2).

This modified procedure consisted of transcervical insertion of a polyvinylchloride (PVC) bar (diam. 3 mm; length 1 m) with a hooked top (1 cm) into the uterus by pressing and rotating the bar carefully along the cervical folds (Fig. 1A). A polyethylene (PE) tube (diam. 5 mm; length 70 cm) was modified by bending the top (2 cm) approximately 450 and fitting an inflatable latex cuff next to the bent top. A straight PVC bar (diam. 3 mm; length 1 m) was used as stylet to straighten the flexible bent top of this tube and to guide the tube along the hooked bar through the cervix into the uterus (Fig. 1B). After insertion of the tube into the uterus, the hooked and the straight bar were removed. The cuff was inflated with 10-15 mL of air and the tube was retracted into the uterine body until the cuff touched the cervix. The tube was rotated to point the bent top in the direction of the left or right uterine horn and a PE catheter (diam 1.5 mm) with several lateral holes near the blunt top was inserted through the tube to flush the horn of choice (Fig. 1C). Each horn was flushed with approximately 100-150 mL of Dulbecco's PBS in portions of 10 mL. by injecting and recovering the fluid trough the same PE catheter. After flushing of both horns, each horn was flushed again with 50-100 mL of PBS. After the flushing procedure 50 mL of diluted Lugol solution (0.15% Iodine and 0.3% Potassiumiodide in distilled water) was infused into the uterine body.

11 Flushings (one to three per sow) were performed with this modified flushing procedure and the number of eggs/embryos flushed was determined. Their development was examined microscopically (40 x magnification) and categorised as unfertilised (1-cell or degenerated) or developing embryos. These sows were slaughtered on Day 5 ± 1 after a fourth insemination to inspect surgical results and to determine number of corpora lutea (CL) and

number and development of the eggs/embryos. Immediately after stunning and bleeding the uteri were obtained and macroscopically examined for surgical results. After removal of the mesometrium the uteri and oviducts were flushed for egg/embryo collection: the resectioned horns with 2 x 20 mL and the oviducts with 2 x 10 mL Dulbecco's PBS. The length of the horns was measured from the utero-cervical to the utero-tubal junction under minimal stretch.

The recovery rate determined after slaughter is based on the total number of collected eggs/embryos and expressed as percentage of the number of CL.

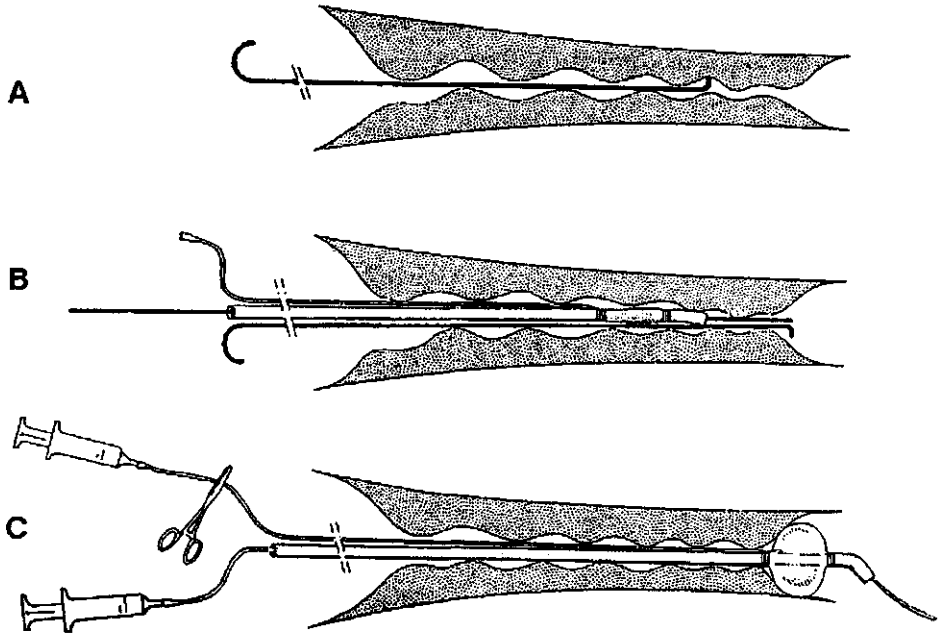

Figure 1. Schematic view of the transcervical flushing procedure.

A: Passage of the hooked bar along the cervical folds.

B: Passage of the bent tube along the hooked bar through the cervix, with guidance of the straight bar as stylet to straighten the flexible top.

C: The bent tube with inflated cuff retracted against the cervix and a catheter introduced for flushing purposes.

Experiment 2

Seven sows with resectioned uteri and six intact control sows were tested for oestrus and inseminated as described in Exp. 1. At Day 5 or 6 after insemination the modified sows were transcervically flushed as in Exp. 1. The embryos from the control sows were collected after slaughter by flushing each uterine horn with 2 x 30 mL PBS.

The number of eggs/embryos per flushing was determined and the eggs/embryos were categorised as U/D (= unfertilised or degenerating, RET (= retarded (2-8 cells)) or M/BL (= morula or blastocyst). Thereafter, embryos were subjected to a hypotonic treatment (10 min. in .6 % KCl solution) and disrupted with methanol/acetic acid (3/1 v/v) on glass slides. The nuclei were stained with Giemsa and counted. The embryonic development was expressed as the ECC (= number of embryonic cell cycles = 2log of the nuclei count).

Experiment 3

Embryos were transcervically collected from the seven sows with resectioned uteri of Exp. 2 in three consecutive oestrous cycles. The first and third oestrous cycle of the sows were not treated. In the second cycle the sows were treated with 2x500 (g cloprostenol (Estrumate®, Pitman-Moore bv, Haarlem, The Netherlands) i.m. with 12 hrs interval on Day 12±1 after previous ovulation, followed by 2000 IU PMSG and 750 IU hCG (Folligonan® and Chorulon®, Intervet bv, Boxmeer, The Netherlands) i.m. 24 and 96 hrs after first cloprostenol injection, respectively. Ovulation was detected by three times daily transrectal ultrasonography (Scanner 150V, Pie Medical Benelux bv, Maastricht, The Netherlands), as described by Soede et al. (1992), for precise determination of the embryonic age at the time of embryo collection. Sows were artificially inseminated as in Exp. 1, but twice each oestrus. Embryos were collected 94-116 h after ovulation and their development was determined as in Exp. 2.

Statistical analyses

Statistical analyses were performed by Students t-test (Exp. 1 and 2) or NPAR1WAY procedure (Kruskal-Wallis test for %U/D, %RET, %M/BL in Exp. 2 and 3) or the GLM procedure (# eggs and ECC after correction for sow and embryonic age in Exp. 3) of SAS (1990). All means are expressed as mean ± SD.

RESULTS

Experiment 1

Results of surgical procedures

After surgery of the sows, three sows were culled and slaughtered. Two sows because of endometritis in one excluded horn (aseptic purulent content) and one sow due to repeated problems with transcervical embryo collection (penetration of the cervical wall).

In the six sows of Exp. 1, approximately 6 months after resectioning, no adhesions between fimbriae and ovaries and only some minor superficial adhesions between different uterine parts were detected after slaughter. No obstructions in the resectioned uterine horns were detected. All excluded horns contained some fluid. The length of the resectioned horns varied between 11.5 and 30.0 cm (average 22.0±5.4 cm).

Cyclicity after resectioning

All sows remained cyclic until slaughter (8-11 cycles). The length of the oestrous cycle in the modified sows was not influenced by surgery (22.0±1.9 versus 20.7±.8, $P>0.05$), while the length of the cycles in which embryos were recovered increased with one day (21.2±1.3 versus 20.2±1.2, $P<0.05$, $n=18$) in comparison to the next cycle without insemination and embryo collection.

Recovery of eggs/embryos

Using the modified transcervical flushing procedure, 6.2±4.2 eggs/embryos per flushing (n=11) were collected (Table 1).

The number of recovered eggs/embryos after slaughter (Table 1) was 12.0±6.0 per sow (excluding one sow with only two embryos resulted in 14.0±3.9 eggs/embryos per sow). The average recovery rate was 62±29% with on average 19.7±2.5 CL per sow (n=6). No eggs/embryos were collected from oviducts.

Embryonic development

Of the eggs/embryos recovered, 80.5% were developing embryos (after omittance of one flushing with all eggs unfertilised), including one sow with 18 of the 19 eggs unfertilised at slaughter (Table 1).

Table 1. The recovery of eggs/embryos collected transcervically (TC) and after slaughter (SL) from sows with a resectioned uterus (Exp. 1).

Sow	1st TC	2nd TC	3rd TC	SL	Recovery Rate (%)
1	T	T	6 (6)	11 (11)	65
2	T	T	1 (1)	2 (2)	9
3	T	7 (7)	7 (0)[a]	13 (11)	76
4	T	0 (0)	9 (3)	10 (10)	53
5	T	5 (5)	1 (1)	19 (1)	86
6	9 (9)	14 (14)	9 (9)	17 (17)	85
Mean		6.2±4.2		12.0±6.0	62±29

T = test procedures to modify the transcervical flushing procedure

( ) between parenthesis the number of developing embryos

[a] including two not categorized eggs

Experiment 2

Recovery of eggs/embryos

The number of transcervical collected eggs/embryos was 6.3±3.4 (n=7) and significantly (P<0.05) lower than the 17.7±2.2 collected from intact sows after slaughter (n=6; Table 2).

Table 2. Average number and development of eggs/embryos per sow, recovered transcervically from modified sows or after slaughter from intact sows (Exp. 2).

	modified sows		intact sows	
# eggs/embryos	6.3±3.4[b]	(7)	17.7±2.2[a]	(6)
% U/D	15.0±19.1	(4)*	5.9± 6.9	(6)
% RET	0.0±0.0	(4)	13.5±33.2	(6)
% M/BL	85.0±19.1	(4)	80.5±39.6	(6)
# ECC	4.0±0.9	(4)	4.2±1.5	(6)

[a,b] means with different superscripts within rows are different (P<0.005)

( ) between parentheses number of flushings

* excluding one flushing with no eggs and two flushings with all eggs unfertilized

Embryonic development

The % M/BL per sow was 85.0±19.1% (n=4; after omittance of one flushing with no eggs and two flushings with all eggs unfertilised) and 80.5±39.6% (n=6) in the modified and the intact sows, respectively (P>0.05). The average embryonic development (ECC) of

transcervically collected embryos was 4.0±0.9 (n=4) and not significantly different from 4.2±1.5 (n=6) cell cycles of embryos collected from intact sows after slaughter (Table 2).

Experiment 3

Recovery of eggs/embryos

In the treated second cycle, ultrasonography indicated the occurrence of superovulation in all sows. On average the number of collected eggs/embryos were 8.1±5.5, 18.4±25.0 and 8.0±6.3 in Cycle 1, Cycle 2 and Cycle 3, respectively. These differences were not significant (P>0.05; Table 3).

Table 3. Average (±SD) number* and development** of collected eggs/embryos per sow after superovulation induction in Cycle 2 or in the control Cycles 1 and 3 (Exp. 3).

	Cycle 1	(n)	Cycle 2	(n)	Cycle 3	(n)
# eggs/embryos	8.1±5.5	(7)	18.4±25.0	(7)	8.0±6.3	(5)
(range)	(4-19)		(0-71)		(1-18)	
% U/D	4.4±6.2	(5)	15.6±8.3	(5)	8.3±14.4	(3)
% RET	6.7±14.9[c]	(5)	24.7±20.2[c]	(5)	1.9±3.2[c]	(3)
% M/BL	89.0±13.7	(5)	59.7±26.3	(5)	89.8±13.1	(3)
ECC	4.4±0.7[a]	(5)	3.3±0.6[b]	(5)	3.5±0.4[b]	(3)

* excluding an unsuccessful flushing and a non-ovulating sow, both in Cycle 3

** excluding two flushings in each cycle without fertilised embryos or no eggs/embryos at all

( ) number of flushings

[a,b] means with different superscripts within a row differ, P<0.01.

[c] significant effect of cycle, P<0.05.

Embryonic development

In two flushings of each cycle only unfertilised eggs were collected. In the remaining flushings the embryonic development was significantly affected by the hormone treatment (Table 3). %RET was significantly increased in the treated cycle (6.7±14.9, 24.7±20.2 and 1.9±3.2 in Cycle 1, 2 and 3 respectively; P<0.01) and %M/BL was 89.0±13.7, 59.7±26.3 and 89.8±13.1 in Cycle 1, 2 and 3, respectively (P>0.05). In comparison to the first untreated cycle, ECC, after correction for effects of sow (P=0.07) and embryonic age (hours between ovulation and embryo collection; P<0.01) was significantly decreased in the treated and subsequent untreated cycle (4.4±0.7, 3.3±0.6 and 3.5±0.4 in Cycle 1, 2 and 3 respectively; P<0.01).

DISCUSSION

After uterine resectioning two sows were excluded because after surgery endometritis in one excluded horn occurred. It might be speculated that this was due to surgical trauma, since no bacterial contamination was detected. Additionally, one sow was excluded because repeated cervical passage problems occurred. In total 13 out of 16 sows (81%) were useful for transcervical flushing and embryos were collected repeatedly from these sows.

After slaughter of the modified sows (Exp. 1), no ovarian adhesions were detected and the suggestion in a previous study (Hazeleger et al. 1989) that these adhesions might be responsible for low recoveries, could therefore not be substantiated. Whether the fluid in the excluded horns disturbs reproductive processes is not yet clear, but the present results show no major effects. All slaughtered modified sows had a normal number of CL and in most modified sows a normal number of eggs/embryos was present after slaughter.

No indications of disturbance of the cyclicity of the sows, due to the uterine resectioning were detected (Exp. 1). Additionally, the seven sows of Exp. 2 and 3 remained cyclic during more then 2 years (unpublished results), even after insemination without subsequent embryo collection. The results of Kobayashi et al. (1989) showed that anoestrus had occurred in all modified sows after one to nine oestrous cycles, probably due to persistency of corpora lutea since treatment with prostaglandines caused reoccurrence of oestrus. This anoestrus might be due to the removal of the excluded horns, since partial hysterectomy results in persistency of corpora lutea depending on the amount of uterus removed (Anderson et al. 1961, Du Mesnil Du Buisson 1961, Pope et al. 1987). In our study the excluded parts of the horns were left in situ. The length of the oestrous cycle was hardly affected by the flushing procedure. The increase of one day in Exp. 2 must be due to insemination, short term pregnancy (5 days) and/or stimulation of the uterus or cervix by the flushing procedure or the Lugol solution infused. Stimulation of uterus or cervix in cow and mare and surgical stress in pigs also have been found to affect oestrous cycle length (Gilbert 1989, Coubrough, 1985, Hennessy and Williamson 1983). However, the results as found in pigs (Hennessy and Williamson 1983) give no explanation for the one day increase in cycle length in our experiment.

The recovery rate of eggs/embryos after slaughter of the modified sows was 62±29%. One sow had an extreme low recovery (2 embryos), but in the other sows a recovery rate of 73±14% was obtained. The uterus of the sow with an extreme low recovery after slaughter

was macroscopically not different from the uteri of the other modified animals. It is not clear whether flushing of the resectioned uterus is less efficient than flushing of normal uteri.

The modified transcervical collection procedure resulted in a recovery of 6.2±4.2, 6.3±3.4, 8.1±5.5 and 8.0±6.3 eggs/embryos in Exp. 1, 2 and 3 (Cycle 1 and 3), respectively. After superovulation induction the recovery is on average 18.4±25.0 eggs/embryos (range 0-71). These results show a large variation in recovery. Superovulation induction is known to give very variable responses (Holtz and Schlieper 1991). The recovery results of Kobayashi et al. (1989) are comparable to our results, although they used a Foley-catheter with manual guidance per rectum to flush the individual horns. However, manual guidance per rectum is not always possible and a selection of sows beforehand is therefore necessary (Kobayashi et al. 1989). The advantage of our flushing procedure is that no manual guidance per rectum is needed.

The frequency of flushings with only unfertilised eggs (excluding flushings with no eggs) was 6% (1/16), 33% (2/6) and 28% (5/18) in the modified sows of Exp. 1, 2 and 3, respectively. This means that 80% (32/40) of the flushings contained fertilised eggs/embryos.

The embryonic development in sows with resectioned uteri was similar to that in intact sows (Exp. 2). This is in agreement with earlier results reported, which indicated that in sows, in which uterine space was restricted, no altered development or survival of embryos during early pregnancy occurred (Dziuk 1968, Fenton et al. 1970, Webel and Dziuk 1974). Superovulation induction (Exp. 3) caused a retardation in morphological development of the embryos and a reduction in number of embryonic cell cycles. This supports the observation of poor embryonic development and poor transfer results after superovulation (Holtz and Schlieper 1991). No clear explanation can be given for the reduction in embryonic cell cycles in the control cycle after superovulation induction, however, only three observations were available.

In conclusion, this study shows that uterine resectioning gives the opportunity for repeated transcervical embryo collection without causing reproductive disorders. In order to prevent anoestrus in animals with resectioned uteri, the excluded parts of the uterine horns should be left in situ. A normal number of eggs/embryos is present in most sows with a resectioned uterus and there are no indications of impaired embryonic development. Superovulation induction can increase the recovery, but the results are very variable. A further increase in number of collected eggs/embryos might be realised by finding the cause of, and a remedy for, the low recovery rates in some flushings.

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Chapter 3

FARROWING RATE AND LITTER SIZE AFTER TRANSCERVICAL EMBRYO TRANSFER IN SOWS

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Published in:
Reproduction in Domestic Animals 29 (1994) 481-487
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FARROWING RATE AND LITTER SIZE AFTER TRANSCERVICAL EMBRYO TRANSFER IN SOWS

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ABSTRACT

A transcervical embryo transfer procedure was evaluated in two experiments with synchronised donor and recipient sows. In Experiment 1, the recovery of the transferred embryos was determined 3 h after transfer. In Experiment 2, farrowing rate and litter size were determined. In Experiment 1 in six sows, the average (mean ± SD) recovery of the transferred embryos was 74±18%. In Experiment 2 (n=21), farrowing rate was 33%. When the transfers were differentiated according to the developmental stage of the transferred embryos (morulae in the presence (n=11) or absence (n=10) of blastocysts), the pregnancy rate was 55% (6/11) in the case of transfers with blastocysts and 10% (1/10) for transfers with only morula stage embryos (P=0.06). Litter size was 6.7±1.6. Of the 14 recipients which were not pregnant, six showed a prolonged oestrus interval of 25-29 days. It is concluded that, in this experiment, the pregnancy rate after nonsurgical embryo transfer depended on the developmental stage of the transferred embryos. The possibility of increasing embryonic survival and pregnancy rate by a proper choice of developmental stages of the transferred embryos and/or synchrony of recipients is discussed.

Key words: transcervical, embryo transfer, pig

INTRODUCTION

Previous research (Polge and Day 1968, Sims and First 1987, Hazeleger et al. 1992, Mödl et al. 1993, Reichenbach et al. 1993, Galvin et al. 1994) has shown that non-surgical embryo transfer in pigs is possible. Transcervical embryo transfer procedures have resulted in low pregnancy rates up to foetal stages or up to birth (10-20%; Polge and Day 1968, Reichenbach et al. 1993, Galvin et al. 1994) and needed either mild (Polge and Day 1968, Sims and First 1987, Galvin et al. 1994) or complete anaesthesia (Reichenbach et al. 1993, Mödl et al. 1993). Hazeleger et al. (1992) showed that transcervical intrauterine deposition of embryos is possible using a small volume of medium and without the need for sedation of the sows. Short-term (2-day) embryonic development after transfer appeared to be normal (Hazeleger et al. 1992).

The aim of this study was to determine pregnancy rate and litter size by using a slightly modified transcervical embryo-transfer procedure.

MATERIAL AND METHODS

Animals

53 cyclic, multiparous, commercial crossbred sows (Dutch Landrace x Great Yorkshire) were used as embryo donors (n=26) or embryo recipients (n=27). Oestrus was checked once daily using the standing response to a boar. The estrous cycle of the animals was synchronized by induction of luteolysis with 2x500 µg cloprostenol (Estrumate®; Pitman-Moore Nederland bv, Haarlem, The Netherlands) i.m. at 8 h intervals on Day 13, 14, 15 or 16 of the cycle (Day 0 = first day of oestrus), followed by 1000 iu PMSG (Folligonan®; Intervet Nederland bv, Boxmeer, The Netherlands) i.m. 24 h after the first cloprostenol injection and 1000 iu hCG (Chorulon®; Intervet Nederland bv, Boxmeer, The Netherlands) i.m. 96 h after the first cloprostenol injection. Donors were inseminated at 22 h and 30 h after hCG using a commercial A.I. dose ($3x10^9$ sperm cells). All animals were checked for ovulation using transrectal ultrasonography (Scanner 150S, Pie Medical bv, Maastricht, The Netherlands) at approximately 50 h after hCG. Only sows that showed signs of oestrus and had ovulated within approximately 50 h after hCG were included in the experiments to prevent a large asynchrony between animals.

Embryo collection and transfer

Donors were slaughtered at a local slaughterhouse on Day 6 (144 h) after hCG. Immediately after stunning and bleeding, the reproductive tract was removed and flushed with Dulbecco's PBS (Sigma, USA) and 1% lamb serum (Gibco, UK) at 39 °C. Morphologically normal embryos (morulae/blastocysts) were collected and placed in transfer medium (Dulbecco's PBS plus 10% lamb serum; 39 °C) in 0.25 mL straws. After transport (39 °C), 14-21 embryos of each donor were washed three times in transfer medium (39 °C) and collected in 0.1 mL transfer medium in the top of a teflon embryo catheter (diameter 1 mm), connected to a 1-mL syringe. One flush of 33 embryos was split for two recipients and, in two transfers, a few embryos from another donor were added. The embryos were transferred to a synchronous recipient within 2-3 h after slaughter (146-147 h after hCG).

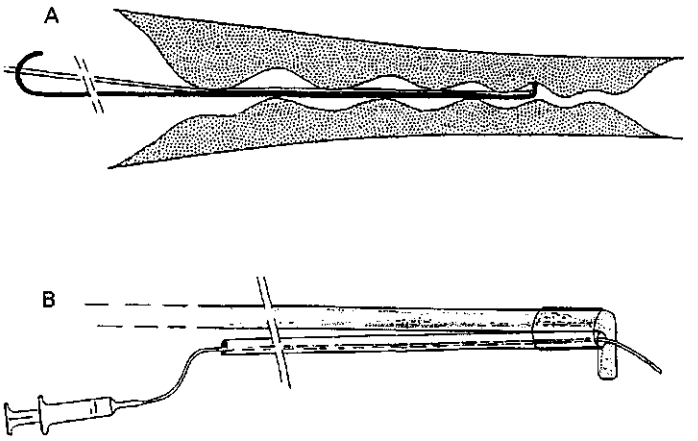

Figure 1: Schematic view of the transfer procedure.
A: Passage of the hooked PVC rod (diam. 3 mm) with connected guiding tube (diam. 2.4 mm), through the cervical folds.
B: Catheter (diam. 1 mm), containing the embryos in 0.1 mL medium, inserted, through the guiding tube, into the uterine body.

Transfer procedure

The transfer procedure (Fig. 1) consisted of the careful passage of a PVC rod (length 1 m; diameter 3 mm) with a 90° hooked top of 1 cm and a side by side connected teflon guiding tube (length 1 m; diameter 2.4 mm) through the cervical folds into the uterine body. The instrument is protected by a polyethylene sheet until approximately halfway through the cervix, to prevent contamination of the uterus. The embryo catheter (diameter 1 mm), containing the embryos in the tip, is passed through the guiding tube into the uterine body and the embryos are injected into the uterine body in 0.1 mL transfer medium.

Experiment 1

In Experiment 1, six recipients were slaughtered at approximately 3 h after embryo transfer to recover the embryos. The uterine horns were flushed with Dulbecco's PBS immediately after stunning and bleeding. The uterus was checked for damage caused by the transfer procedure.

Experiment 2

In experiment 2, 21 recipients were checked for oestrus each day after transfer and, from Day 14 after transfer onwards, occasionally checked for pregnancy by transrectal and transcutaneous ultrasonography, until farrowing.

Statistical analysis

All means are expressed as mean ± SD and pregnancy rates are analysed by Fisher's Exact Test using the FREQ procedure of the SAS package (SAS, 1990).

RESULTS

Experiment 1

In Experiment , the recovery of embryos immediately after transfer was 12.5±6.2 of 17.0±6.4 transferred embryos (74±18%; n=6; table 1). One sow had superficial endometrial damage and a relatively low recovery (42%) of transferred embryos. Omitting this sow resulted in a recovery of 80.2±9.2% of transferred embryos.

Table 1. Recovery of embryos immediately after transfer.

Transfer no.	Embryos transferred (n)	Embryos recovered (n)	Recovery Rate (%)
1	10	9	90
2	15	10	67
3	12	9	75
4	28	24	86
5[1]	19	8	42
6	18	15	83
Mean ± SD	17.0 ± 6.4	12.5 ± 6.2	74 ± 18
			80 ± 9[2]

[1] Superficial damage to endometrium

[2] Flushing no. 5 excluded

Experiment 2

In Experiment 2, 20±7 embryos were recovered on average from the donor sows (81±10% of the corpora lutea count). The transfer of 14-21 (17±2) embryos per recipient to 21 recipients resulted in 7 pregnancies (33%; Tables 2 and 3). Of the 14 recipients which returned to oestrus, eight showed a normal oestrous cycle length of 19-24 days. The other six recipients had foetal fluid in the uterus and/or corpora lutea on Day 20 after hCG and returned to oestrus between Day 24 and 30 after hCG.

Table 2. Classification of the transferred embryos and the resulting litter size or length of the oestrous cycle after transfer (Exp. 2).

	Transferred embryos (n)					
Recipient no.	Morulae	Comp. Mor.	Blastocyst	Total	Piglets Born (dead)	Cycle length (d)
1	0	17	4	21	9 (1)	-
2	0	9	11	20	8 (2)[3]	-
3	0	7	10	17	7 (0)	-
4[2]	9	7	0	16	7 (0)	-
5	0	3	12	15	7 (1)	-
6	4	5	8	17	5 (1)	-
7	2	12	4	18	4 (0)[4]	-
8	5	11	0	16	-	29[1]
9	0	1	17	18	-	29[1]
10	5	15	0	20	-	27[1]
11	6	7	3	16	-	26[1]
12	8	7	0	15	-	25[1]
13	0	12	6	18	-	25[1]
14	8	4	7	19	-	24
15	14	3	0	17	-	23
16	5	10	0	15	-	22
17	5	14	0	19	-	22
18	13	2	0	15	-	21
19	7	7	0	14	-	21
20	7	10	0	17	-	19
21	3	3	13	19	-	19
Mean ± SD	4.8 ± 4.1	7.9 ± 4.4	4.5 ± 5.3	17.2 ± 1.9	6.7 ± 1.6	23.4 ± 3.3
n	21	21	21	21	7	14

[1] Intrauterine embryonic material and/or corpora lutea detected by transrectal ultrasonography on Day 20 after hCG.

[2] Litter without blastocysts recruited out of Litter 3 with blastocysts.

[3] Including 1 mummified foetus.

[4] Additionally 2 degenerating placentas were found.

The presence of pre-compaction morulae in the transferred embryos resulted in extended oestrous cycles (>24 days) in 7 out of 15 recipients, while absence of pre-compaction morulae in the transferred embryos resulted in extended oestrous cycles (>24 days) or pregnancies in all six of the recipients ($P<0.05$; Table 2).

If blastocysts were among the embryos transferred (11 litters), six recipients farrowed (55%), whereas transfers without blastocysts (10 litters) resulted in only one farrowing (6 out of 11 vs 1 out of 10; P=0.06; Table 3). The transfer without blastocysts leading to a pregnancy consisted of embryos that were recruited out of a flush containing 10 blastocysts (Table 2).

Litter size at farrowing was 6.7±1.6 (38±8% of the transferred embryos; n=7). Of the 47 piglets born, one was mummified and four were stillborn. Additionally, two degenerating placentas without fetal tissue were recovered from a recipient with four piglets born. The average of birthweight per litter was 1580±147 g.

Table 3. Summary of transfer results for litters with or without blastocysts (Exp. 2).

	No. of transfers		
Recipients	With blastocysts	Without blastocysts	Total
Total	11	10	21
Pregnant	6 (55%)	1 (10%)[1]	7 (33%)
With elongated cycle[2]	3 (27%)	3 (30%)	6 (29%)
Non-pregnant	2 (18%)	6 (60%)	8 (38%)

[1] Morulae originating from a flushing containing blastocysts

[2] Intrauterine embryonic material and/or corpora lutea detected by transrectal ultrasonography on Day 20 after hCG and estrous cycle > 24 days

DISCUSSION

The results show that it is possible to obtain pregnant recipients with a rather simple transcervical embryo transfer procedure. The 80% embryo recovery shortly after transfer, as found in Experiment 1, is comparable with embryo recoveries achieved by slaughter of the donor sows (81±10%), indicating that the transfer procedure is adequate. Damage of the endometrium was observed in one case (Transfer 5; Experiment1) and was accompanied by a relatively low embryonic recovery after slaughter. In a preliminary study (Hazeleger; unpublished data) it was found that a transfer instrument that was introduced too far into the uterus caused superficial scratching on the endometrium. This caused little or no bleeding and was always accompanied by a low recovery (37±23%; n=9) of the transferred embryos after slaughter.

Farrowing rates are affected by the development of the transferred embryos, although all transfers were performed in synchronized donor-recipient couples. The presence of pre-compaction morulae in the transferred embryos resulted in normal estrous cycles in 8 out of 15 recipients, while absence resulted in elongated cycles or farrowings, indicating a possible poor developmental potency in these embryos. The presence or absence of blastocysts in the transferred embryos resulted in 55% and 10% farrowing, respectively. The transfers which did not result in pregnancies often resulted in estrous cycles of 25-29 days. Return to oestrus around Day 27 also occurs when embryos are removed after elongation (Van der Meulen et al. 1988). During elongation estrogens are produced, which are needed for initial rescue of the corpora lutea. After elongation the presence of differentiating embryos is needed to prevent return to oestrus around Day 27 (Van der Meulen et al. 1988, Geisert et al. 1990). It might be speculated that development after elongation is disturbed by the transfer conditions. These conditions might disturb the process of compaction and blastulation (formation of inner cells) at the morula stage, resulting in poor development after elongation. Since morula stage embryos were present in all transfers, it might also explain the relatively high losses in the pregnant recipients. Litter size at birth was 6.8, while 15-21 embryos were transferred. There are no indications that the transfer conditions in this study are any different from established collection conditions (embryo collection by slaughter: Schlieper and Holtz 1986) or surgical-transfer conditions (medium and temperature: Schlieper and Holtz 1986, Cameron et al. 1989, Niemann et al. 1989). The only essential difference to the established procedures was the transcervical deposition of the embryos in the uterus near the cervical junction. The location of morulae and early blastocysts in the uterus does not seem to be very critical since transfer to the middle region of the uterus has also been successful (Stein-Stefani and Holtz 1987). However, culture conditions and even minor differences in medium composition affect embryonic development (Hyttel and Niemann 1990, Blum-Reckow and Holtz 1991, Beckmann and Day, 1993) and the effects of in vitro culture over a few h cannot, therefore, be excluded.

The realized litter sizes (6.7) and farrowing rate in the case of transferred blastocysts (55%) are comparable to the average results of surgical transfers (6.5 and 58%, respectively; Brüssow and König 1990). The advantage of transferring blastocysts might be reflected by the better results of Mödl et al (1993), with a pregnancy rate of 55% after non-surgical transfer of blastocysts to nine synchronous and two asynchronous recipients, compared with their previous results showing a pregnancy rate of 10% with varying developmental stages (Reichenbach et al. 1993). This improvement may, however, be due to the modified transfer procedure. The results of this study indicate a relatively high survival rate during the first week after transfer (82% when only litters with blastocysts are transferred) and high embryonic mortality thereafter, based

on an extended cycle length in 27% of these recipients. The results of Sims and First (1987), who transferred blastocysts, also show high pregnancy rates 1 week after transfer.

The improved results obtained after transferring blastocysts in our experiment might also reflect a slight asynchrony between donor and recipient in some cases. Some asynchrony between animals can be expected, since the exact moment of ovulation was not determined; only animals which ovulated within 50 h after hCG administration were used. Among the synchronized donors, variation in embryonic development occurred, indicating a lack of synchrony among these animals. A similar lack of synchrony must exist among the recipients. Consequently, this resulted in transfers in which blastocysts were transferred synchronously or with a developmental advantage, while less developed embryos were transferred synchronously or with a developmental disadvantage. Asynchronous transfer with a developmental advantage for embryos usually leads to improved results (Polge 1982), especially in the case of transferring embryos with a developmental disturbance due to culture conditions (Blum-Reckow and Holtz 1991). A more accurate estimation of synchrony between donors and recipients is needed in order to find the cause of the better survival of blastocysts.

It can be concluded that, in this experiment, the farrowing rate after transcervical embryo transfer depended on the developmental stage of the transferred embryos and, possibly, on a slight asynchrony between donors and recipients. The pregnancy rate and litter size might be improved by a correct choice of the developmental stages of embryos and/or asynchrony between donors and recipients.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the participating students for their assistance, as well as T. van der Lende and N.M. Soede for their contribution to the manuscript.

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Chapter 4

EFFECT OF ASYNCHRONOUS NON-SURGICAL TRANSFER OF PORCINE EMBRYOS ON PREGNANCY RATE AND EMBRYONIC SURVIVAL

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Submitted for publication to:
Livestock Production Science

EFFECT OF ASYNCHRONOUS NON-SURGICAL TRANSFER OF PORCINE EMBRYOS ON PREGNANCY RATE AND EMBRYONIC SURVIVAL

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ABSTRACT

Embryo survival was determined after non-surgical transfer to recipients with a variable synchrony of ovulation. Groups of 10 to 15 freshly weaned multiparous sows (donors and recipients) were checked for time of ovulation and paired, resulting in recipients ovulating 24 h before the donors up to recipients ovulating 36 h after the donors (asynchrony –24 to +36 h). Embryos were collected from 34 donors at 120 h (range 108-132 h) after ovulation and 16.6±2.4 morulae and blastocysts were transferred to 31 recipients. Pregnant recipients were slaughtered on Day 35 (Day 0 = ovulation) to evaluate embryonic survival and development.

Twelve recipients were pregnant at Day 21 and 5 were still pregnant at Day 35. An asynchrony of +18 h to +36 h resulted in 1/12 recipients pregnant at Day 21 and no pregnancies at Day 35, while an asynchrony of -24 h to +12 h resulted in 11/18 recipients pregnant at Day 21 and 5 still pregnant on Day 35 ($P<0.05$). The presence of ≥6 morulae within a litter never resulted in pregnancies at Day 21 (0/9 vs. 12/21 (<6 morulae); $P<0.05$), irrespective of the degree of asynchrony.

The results indicate that at 108 to 132 h after ovulation only blastocysts can be transferred successfully by a non-surgical procedure. Recipients have to ovulate between 24 h before to 12 h after the donors. Transfers to recipients ovulating 18 to 36 h after the donors lead to very low pregnancy rates. However, these conclusions need to be confirmed in a study with more animals.

Key words: pig, embryo transfer, non-surgical, asynchrony, ultrasonography

INTRODUCTION

With the development of non-surgical embryo transfer techniques in pigs a revalidation of some transfer conditions might be needed, since these are based on surgical transfers. The average results of the current non-surgical procedures (Reichenbach et al. 1993, Galvin et al. 1994, Hazeleger and Kemp 1994, Li et al. 1996, Yonemura et al. 1996), range from 9 to 60% farrowing rate and 3.1 to 6.7 piglets born. These results are less than the average results of surgical procedures with a farrowing rate of 58% and a litter size of 6.5 (Brüssow and König

1988). One of the major differences between surgical and non-surgical procedures is the location of the embryos immediately after transfer. In non-surgical procedures the embryos are deposited in or near the uterine body, instead of deposition in the top of the uterine horns used in surgical procedures. Using surgical transfers, embryos on Day 5 after ovulation have equal chances to survive in the middle or in the top of the horns (Stein-Stefani and Holtz 1987), but expanded blastocysts have a reduced survival after transfer to the caudal region of the horns and very poor survival after transfer to the uterine body (Wallenhorst and Holtz 1995).

Previous research with our non-surgical transfer procedure indicated a higher survival of blastocysts compared to morulae in synchronous transfers at Day 4 (Hazeleger and Kemp 1994). It might be that blastocysts are in a more appropriate stage of development for survival in the environment of the uterine body. However, in that study the better survival of the blastocysts might also have been caused by the relative advanced development of the blastocysts or retarded development of the morulae in comparison to the development of the uterus of the recipient. In previous research using surgical transfer, embryos with an advanced development showed a better survival (Polge 1982). Such asynchrony (embryos ahead of uterine environment) might also favour the survival of the transferred embryos when embryonic development is impaired due to culture conditions (Blum-Reckow and Holtz 1991) or the survival of the smaller littermates within a litter (Wilde et al. 1988). It seems plausible that also in non-surgical transfers embryos may profit from a certain degree of asynchrony between donor and recipient as shown for surgical transfers (Polge 1982). Therefore, in this study, embryos recovered 120 h (range 108 - 132 h) after ovulation were transferred to recipients with a variable synchrony of ovulation, to find an optimal synchrony window for non-surgical transfers.

MATERIALS AND METHODS

Animals

Groups of 10 - 15 commercial crossbred (Landrace x Yorkshire) sows arrived at the experimental station at the day of weaning (approx. 3-4 weeks after farrowing). Oestrus was checked once daily by the standing response in front of a boar. Donors were artificially inseminated at the first and second day after onset of oestrus, with a commercial dose of 80 mL mixed semen (3×10^9 cells) of three different Yorkshire boars.

The time of ovulation of all animals was estimated by transrectal ultrasonography (Scanner 150S, with a 5.0 mHz MAP-transducer, Pie Medical bv, Maastricht, The Netherlands)

starting 12 h after onset of oestrus, repeated every 6 (20 transfers) or 12 h (11 transfers), until ovulation had taken place.

Asynchrony was based on the difference in time between ovulation of recipients and donors. This resulted in recipients ovulating 24, 18, 12, 6 or 0 h before or 6, 12, 18, 24, or 36 h after the time of ovulation of donors. The 6 or 12 h intervals between ultrasonography lead to a synchrony reliability range of ±3 and ±6 h for each synchronous and ±6 and ±12 h for each asynchronous transfer respectively. Thirty-four donors and 31 recipients, which showed approximately simultaneous oestrus expression and ovulation, were selected and paired

Embryo collection and transfer

The donors were slaughtered at 108 to 132 h after estimated ovulation. Immediately after stunning and bleeding the embryos were collected with Dulbecco's PBS (Sigma, USA) + 1% lamb serum (Gibco, UK) at 39 °C. Embryos were evaluated and morphologically normal embryos (at least morulae stage) were collected and placed in transfer medium (Dulbecco's PBS + 10% lamb serum, 39 °C) in 0.25 mL straws. After transport (39 °C), embryos of each donor were washed three times in transfer medium (39 °C). To ensure a normal physiological variation in embryonic development, embryos from one donor were transferred to one recipient. The goal was to transfer 15 to 20 morphologically normal embryos per recipient. However, when the number of collected embryos was limited a few embryos of similar development from another donor were added (n=7). The embryos were collected in the top of a Teflon embryo catheter (diameter 1 mm), connected to a 1-mL syringe. The embryos were transferred within 2-3 hours after collection.

The transfer procedure (as previously described, Hazeleger and Kemp 1994) consisted of the careful passage of a PVC rod (length 1 m; diameter 3 mm) with a 90° hooked top of 1 cm through the cervical folds into the uterine body. This instrument was connected side by side with a Teflon guiding tube (length 1 m; diameter 2.4 mm). A polyethylene sheet protected the instrument until approximately halfway through the cervix, to prevent vaginal contamination. The embryo catheter (diameter 1 mm), containing the embryos in the tip, was passed through the guiding tube into the uterine body and the embryos were deposited in the uterine body or beginning of a uterine horn with 0.1 mL transfer medium. The recipients were not sedated during transfer.

Recipient data collection

The recipients were checked for oestrous symptoms daily. From two weeks after transfer onwards the recipients were checked with ultrasonography for presence of developing embryos

(embryonic fluid) and corpora lutea three times weekly. Recipients with embryonic fluid and corpora lutea around Day 21 (Day 0 is day of ovulation) and not returning to oestrus before Day 24 were considered as pregnant on Day 21. On Day 35 after ovulation the pregnant recipients were slaughtered to evaluate the survival and development of the transferred embryos. The number of normal foetuses and number of embryonic or foetal remnants (degenerating foetuses and remnants of embryonic or placental tissue) was counted.

Statistical analysis

All data are expressed as mean ± SD (and range). After post-priory splits of synchrony intervals between donors and recipients, pregnancy rates were analysed by Chi-square analysis or Fisher's Exact Test using the FREQ procedure of the SAS package (SAS 1990).

RESULTS

Embryos transferred

On average 16.6±2.4 (12-20) embryos were transferred to 31 recipients. The transferred litters consisted of on average 3.5±5.5 (0-20) morulae, 6.1±6.3 (0-20) blastocysts, 6.5±6.3 (0-19) expanded blastocysts and 0.5±1.4 (0-5) hatched blastocysts from 34 donors.

Pregnancy rate

One of the recipients had cystic ovaries (detected on Day 21 after ovulation) and was therefore excluded from analysis. Twelve of the 30 recipients (40%) were pregnant at Day 21 after ovulation. Five of these recipients were still pregnant at slaughter on Day 35 and the other seven recipients returned to oestrus between Days 24 and 35 after ovulation (Table 1).

Asynchrony

By categorising the unsuccessful and successful asynchrony periods, as presented in Table 1, it appears that an asynchrony window of +18 h to +36 h (recipients ovulating after the donors) resulted in only one pregnancy at Day 21 (1/12; 8%) and no pregnancies at Day 35, while an asynchrony window of –24 h to +12 h resulted in 11 pregnancies at Day 21 (11/18; 61%) and five pregnancies at Day 35 (5/18; 28%) $p < 0.05$; Table 1). Within this asynchrony window no differences in pregnancy rate could be detected, although at +12 h the largest litter sizes were found (Table 2).

Table 1. Number and development of transferred embryos and asynchrony (time of ovulation of recipients from 24 h before (-24) to 36 h after (+36) time of ovulation of donors) resulting in recipients with a normal oestrous cycle (Cycle ≤ D24), pregnant at Day 21 (Pregnant D21) or pregnant at Day 35 (Pregnant D35).

	Number of transferred embryos		
Asynchrony	Cycle ≤ D24	Pregnant D21	Pregnant D35
-24		18 (1+8+9+0)[a]	
		14 (0+1+13+0)	
-18	20 (6+6+8+0)		20 (1+6+13+0)
-12	12 (0+0+11+1)		16 (3+13+0+0)*
	15 (7+8+0+0)		
- 6	14 (0+0+14+0)		20 (0+16+4+0)
	18 (18+0+0+0)		
0		17 (0+17+0+0)	
		17 (0+0+12+5)*	
		17 (2+15+0+0)	
+ 6		19 (0+11+8+0)	
+12	19 (0+0+19+0)		14 (2+0+12+0)*
	15 (0+0+12+3)		20 (0+20+0+0)*
+18	17 (1+16+0+0)		
	17 (9+6+2+0)		
+24	16 (0+8+7+1)	14 (0+0+14+0)	
	20 (20+0+0+0)		
	16 (0+2+14+0)		
	13 (9+4+0+0)*		
	15 (0+0+15+0)*		
	19 (11+8+0+0)*		
	13 (0+0+8+5)*		
+36	16 (8+8+0+0)*		
	19 (8+11+0+0)*		

[a] Between parenthesis number of morulae + blastocysts + expanded blastocysts + hatched blastocysts.

* Asynchrony based on ovulation control with 12 h intervals.

Table 2. Asynchrony (time of ovulation of recipients after time of ovulation of donors), number of transferred embryos and, on Day 35 of pregnancy, the number of implantations and foetuses of the pregnant recipients

Recipient	No. of embryos	Asynchrony (h)	No. of implantations	No. of foetuses
1	20	-18	5 (25)[a]	3 (25)
2	16	-12	5 (31)	5 (19)
3	20	-6	8 (40)	3 (15)
4	20	+12	11 (55)	8 (50)
5	14	+12	11 (79)	10 (57)
Mean ± SD	18.0 ± 2.8		8.0±2.7 (46±22)	5.8±2.8 (33±19)

[a] Between parenthesis the percentage embryo survival.

Embryonic development

The presence of 6 or more morulae within a litter of transferred embryos did not result in any pregnancies at all (0/9 vs. 12/21(<6 morulae); $P<0.05$; Table 1). Even the transfers with an asynchrony of +18 h to +36 h (recipients ovulating after the donors, thus a relative advanced development of the embryos) did not result in pregnancies when 6 or more morulae within a litter were transferred. This effect of embryonic development on pregnancy rate was not due to embryonic age since the pregnancy rate at Day 21 of older (>120 h) embryos was not higher then the pregnancy rate of younger (<120 h) embryos (3/10 and 9/20, respectively; $P>0.10$). The addition of some embryos from other donors to small litters had also no effect on the pregnancy rate at Day 21 (4/7 pregnant with added embryos and 8/23 pregnant without added embryos, $P>0.10$).

Embryonic survival

On average 8.0±2.7 implantation sites and 5.8±2.8 normal developing foetuses were found in the 5 pregnant recipients. As percentage of the transferred embryos 46±22% implantation sites and 33±19% normal developing foetuses were found (Table 2).

DISCUSSION

Asynchrony

Despite a low number of pregnant recipients, the results of this study indicate that an asynchrony window of -24 to +12 h (recipients ovulating from 24 h before to 12 h after donors) is optimal for non-surgical transfers. This window resulted in a pregnancy rate at Day 21 of 61% (11/18) and a pregnancy rate at Day 35 of 28% (5/18); after exclusion of litters with more than 6 morulae (which did not result in pregnancies) the pregnancy rate was 73% (11/15) at Day 21 and 33% (5/15) at Day 35.

Embryonic mortality

The large difference between the number of pregnancies on Day 21 (11/15) and on Day 35 (5/15) within the optimal asynchrony window indicates a high rate of embryonic mortality after implantation. This is substantiated by the difference between number of implantations (mean: 8.0±2.7) and the number of normal developing foetuses (mean: 5.8±2.8) in the animals which were still pregnant at Day 35. Also in a previous study (Hazeleger and Kemp 1994) a relatively high number of recipients (6/21) returned to oestrus between Day 24 and 35. After that time no returns to oestrus occurred. This relatively high rate of late embryonic mortality is probably due to some unknown aspects of the transfer procedure.

Embryonic development

A more advanced development of the embryos (donors) relative to the development of the recipients (+18 to +36 h; n=11) resulted in only one pregnancy at Day 21 (9%) and no pregnancy at Day 35. This is in contradiction to results in surgical porcine transfers, where an advanced embryonic development relative to recipients was beneficial for embryonic survival (Polge 1982). Also Wilde et al. (1988) found an increased embryonic survival after asynchronous transfer of the less developed embryos out of a litter. Under sub-optimal conditions also better results may be expected, when the embryos are transferred to recipients ovulating later than the donors. This is shown by Blum-Reckow and Holtz (1991), transferring in vitro cultured embryos to asynchronous recipients. In our experiment there are no indications that either well developed embryos (blastocysts) or embryos with a relatively retarded development (morulae) at approximately 120 h after ovulation could benefit from recipients ovulating later than donors.

Differences between surgical and non-surgical transfer procedures

Since the requirements for asynchrony and possibly for embryonic development, needed for successful non-surgical transfers differ from those for surgical transfers, they must be attributed to the differences in the transfer procedure. The three main differences between our non-surgical transfer method and surgical transfers are that our embryos are collected by slaughter of the donors, the recipients are not sedated and the embryos are transferred transcervically into the uterine body or into the uterine horns, nearby the uterine body.

Embryos collected immediately after slaughter of the donors have the best in vitro development, compared with embryos collected two hours after slaughter (Wollenberg et al. 1990). Results of surgical transfers of such embryos, collected immediately after slaughter, were similar to the results of transfers of surgically collected embryos (Schliepper and Holtz 1986). It is therefore unlikely that collection of the embryos by slaughter is harmful for further development after transfer.

The effect of sedation of the recipients can not be investigated in surgical transfers and in non-surgical transfers no comparison has been made. In other species like cattle no sedation is needed, so one might expect that also no sedation of recipients is needed in pigs. No sedation might even be beneficial since (recovery from) anaesthesia or surgical trauma can have negative effects on physiological processes (Hennessy and Williamson 1983).

The effect of the location of the transferred embryos has been studied before (Stein-Stefani and Holtz 1987) and no differences in embryonic survival were found between the embryos that were transferred to the middle compared to the top of the horn. Survival after surgical transfer of blastocysts to the uterine body resulted in only 2/17 (12%) pregnancies with 4 & 5 foetuses, while the transfers to the caudal quarter and top of the horn resulted in a 81% (13/16) and 88% (14/16) pregnancy rate with on average 5.6 and 8.2 foetuses respectively (Wallenhorst and Holtz 1995). The poor results of these surgical transfers to the uterine body, which are in agreement with other unpublished results (Yonemura et al. 1996), were even worse than published results of non-surgical procedures, in which the number of piglets born ranged from 1 to 10 and the pregnancy rate ranged from 22 to 64% (Galvin et al. 1994, Hazeleger and Kemp 1994, Li et al. 1996, Yonemura et al. 1996). This might indicate that the poor results of surgical transfers to the uterine body (Wallenhorst and Holtz 1995) are negatively affected by other aspects than just the location of the embryos. However, it can not be excluded that the location of the embryos in the uterine body or beginning of the uterine horn is sub-optimal.

The finding that transfer at approximately 120 h after ovulation of blastocysts resulted in the highest pregnancy rates in comparison with morulae, might be due to the fact that these embryos are nearer to the developmental stage in which they normally migrate through the uterine horns. Therefore they might be less sensitive to the assumed sub-optimal location in the uterine body. On the other hand it is also possible that at this stage of pregnancy (approximately 120 h after ovulation) blastocysts are well developed embryos and morulae are relatively retarded in their development in comparison to their age and therefore less viable. This is in agreement with our previous study (Hazeleger and Kemp 1994) where Day 4 embryos were transferred and pregnancies were only achieved when blastocysts were among the transferred embryos. That also indicated that the further developed embryos of a certain age are, the better the chance of survival in synchronous transfers.

Conclusions

The results of this study seem to indicate that at approximately 120 h after ovulation only blastocysts can be transferred successfully by a non-surgical procedure and the time of ovulation of the recipients has to range approximately from 24 h before to 12 h after the time of ovulation of the donors. Transfers to recipients ovulating 18 to 36 h after the donors appear to result in very low pregnancy rates. However, these conclusions need to be confirmed in a larger study.

AKNOWLEDGEMENTS

We thank the students P. Jenneskens and I. Peeters for their significant contribution to this study and dr. N. M. Soede and dr. T. van der Lende for their critical review of the manuscript.

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Chapter 5

EFFECT OF SUPEROVULATION INDUCTION ON EMBRYONIC DEVELOPMENT ON DAY 5 AND SUBSEQUENT DEVELOPMENT AND SURVIVAL AFTER NON-SURGICAL EMBRYO TRANSFER IN PIGS.

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Accepted for publication in:
Theriogenology (1999)

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ABSTRACT

To evaluate effects of eCG dosage on recovery and quality of Day 5 embryos and on subsequent development and survival after embryo transfer, batches of 5 to 10 donor sows were treated with 1000 or 1500 iu eCG. After ovulation induction with 750 iu hCG (72 h after eCG) and insemination, embryos were collected at 120 h after ovulation. Ovulation rate was lower using 1000 iu eCG (28.5±11.7; n=48) compared to 1500 iu eCG (45.7±20.3; n=32; P<0.0001). Embryonic recovery rate (82.9±16.9%) and percentage expanded blastocysts (56.2±31.4%) were similar (P>0.05). Expanded blastocysts of every batch of sows were pooled in two groups within each eCG treatment, containing embryos from normal ovulating sows (≤25 Corpora Lutea (CL)) or embryos from superovulating sows (>25 CL). Average diameter and number of cells of a random sample of the expanded blastocysts per pool was scored. The average diameter of the blastocysts (160.5±11.5 μm) was not affected by eCG dosage or ovulation rate (P>0.05). The average number of cells per embryo was higher in the 1000 iu eCG group (84.3±15.3) in comparison to the 1500 iu eCG group (70.2±1.9; P<0.05), but similar for normal and superovulating donors within each eCG group (P>0.05). Out of the four groups, litters of 28 to 30 blastocysts were non-surgically transferred to 27 synchronous recipients. Pregnant recipients were slaughtered at Day 37 after hCG treatment to evaluate embryonic development and survival. Pregnancy rate for the 1000 and 1500 iu eCG donor groups was 71% (10/14) and 46% (6/13; P=0.17), respectively. Number of implantations and fetuses for the 1000 iu eCG groups was 12.9±3.0 and 11.1±2.7 and for the 1500 iu eCG groups 14.2±7.0 and 10.5±4.6, respectively (P>0.10). Categorizing the litters of blastocysts to below or above the average embryonic diameter (158 μm) per litter, the pregnancy rate was 43% (6/14) and 77% (10/13; P=0.07), respectively. For the litters with smaller embryos 10.8±5.2 implantations and 8.8±3.4 fetuses were found and for the litters with larger embryos 14.9±3.9 implantations and 12.1±2.9 fetuses were found (P=0.09 and 0.06, respectively). Categorizing the transferred litters to below or above the average number of cells per embryo showed no differences in pregnancy rates or number of implantations and fetuses. It was concluded that eCG dosage affects embryonic development at Day 7 after hCG and this was not due to ovulation rate. Embryonic survival after non-surgical transfer was related to the diameter of the blastocysts.

Keywords: superovulation, embryonic development, non-surgical embryo transfer

INTRODUCTION

Non-surgical embryo transfer can be a practical tool in pig production for transfer of valuable genetic material within and between countries with a minimal risk of disease transmission and reduced costs and welfare problems due to transportation. The results of non-surgical procedures are promising, but seem to depend on the developmental stage of the embryos (Hazeleger and Kemp 1994). Therefore more attention has to be given to the source and quality of the embryos. Up to now no efficient in vitro production system for pig embryos has been described, so embryos for transfer have to be recovered from gilts or sows.

Collection at the slaughterhouse is a practical and reliable way to obtain embryos, as they have a similar quality in comparison to surgically collected embryos (Schlieper and Holtz 1986). In previous experiments it appeared that non-surgical transfers are more successful when embryos are in a relatively more advanced stage of development (Hazeleger and Kemp 1994; Hazeleger et al. 1999). Therefore, selection of embryos might improve the results, but leads to a reduction in the number of available embryos and thus in efficiency. High numbers of embryos are important to make this system more efficient, so superovulation induction might be a solution. Previous research showed that by increasing the eCG dosage the number of embryos can be increased (Brüssow and König 1990), but superovulation in prepubertal gilts (Holtz and Schlieper 1991) and sows (Hazeleger et al. 1994) also increases the number of poor quality embryos. It is, therefore, important to know the effects of superovulation in sows on the quality of the embryos and subsequent survival after transfer.

An experiment was designed to study effects of superovulation, comparing a relatively low dose of eCG (1000 iu) with a relatively high dose (1500 iu), on the development of the embryos at the time of transfer and the consequences for further embryonic development and survival after non-surgical transfer.

MATERIALS AND METHODS

Batches (n=11) of 10 to 20 multiparous commercial crossbred (Landrace x Yorkshire) sows arrived on the experimental station on the day of weaning (approximately 3 to 4 weeks after farrowing). Three days later 750 iu hCG (Chorulon®, Intervet bv, The Netherlands) was administered im for induction of ovulation. Fifteen days later luteolysis was induced by two injections (interval 8 h) of 500 µg cloprostenol im (Estrumate®; Malinckrodt Veterinary bv, The Netherlands). To stimulate follicular development eCG (Folligonan®; Intervet bv, The Netherlands) was injected im 22 h after last cloprostenol injection, 800 iu for recipients and 1000

or 1500 iu for donors. Within a batch the donors were treated with the same dosage. Ovulation was induced 72 h later with 750 iu hCG i.m. The time of ovulation of all animals was monitored by transrectal ultrasonography (Scanner 150S, Pie Medical b.v., Maastricht, The Netherlands) at 0, 24, 36, 42 and 48 h after hCG treatment. Estrus was checked with a vasectomized teasing boar once daily. Only sows showing estrous symptoms and which ovulated between 36 and 48 h after hCG (78%) were used in this experiment. Donors were artificially inseminated at 36 and 42 h after hCG with a commercial dose of 80 mL semen (approximately $2x10^9$ sperm cells) each time. Seven days (162 h) after hCG (114 to 126 h after estimated ovulation), the donors were slaughtered and immediately after stunning and bleeding the embryos were collected in Dulbecco's PBS (Sigma, USA) + 1% heat inactivated lamb serum (Gibco, UK) at 39 °C. The embryos were immediately washed with Dulbecco's PBS (Sigma, USA) + 10% heat inactivated lamb serum (39 °C) and transported to the laboratory in 15 mL of this transfer medium, stored in a thermostatically controlled box of 25 °C. The number of Corpora Lutea (CL) on the ovaries were counted. All transferable embryos (expanded blastocysts) were collected and washed three times at room temperature (approximately 25 °C) with fresh transfer medium. From each batch of donors (6 to 10 donors per batch; 1000 or 1500 iu eCG) the expanded blastocysts were pooled in two groups: normal (≤25 CL) or superovulating (>25 CL) donors. A pool of embryos consisted of 10 to 196 embryos from 1 to 6 donors. All embryos were photographed at 63x magnification to determine diameter. Five to ten randomly chosen embryos of every pool were subsequently processed for determination of the number of cells. This was done after hypotonic treatment (0.6% w/v KCL-solution, 0 °C, 10 min) of each individual embryo. After being placed on a fat free glass slide, they were treated with small droplets of methanol/acetic acid (3/1 v/v) until disruption and spreading of the cells. After drying and staining with 10% Giemsa in phosphate buffered saline, the number of nuclei was counted (magnification x 200).

Litters (1 to 3 per pool) of 28 to 30 embryos were retrieved for transfer and photographed for diameter measurements. The litters were collected in the top of a teflon embryo catheter (diam 1 mm), which was filled with transfer medium and connected to a 1 mL syringe. The transfer procedure (Hazeleger and Kemp 1996, modified according to Hazeleger 1997) consisted of a careful passage of a PVC rod (length 1 m; diam 3 mm) with a 1 cm bend top and a side by side connected teflon guiding tube (length 1 m; diam 2.4 mm) through the cervical folds into the uterine body. When the instrument was introduced in the uterine body or beginning of a uterine horn, the embryo catheter (diam 1 mm) containing the embryos in the tip, was passed through the guiding tube into the uterus and the embryos were deposited (maximally 0.1 mL transfer medium). For sanitary precautions Trimethosulf® premix (trimethoprim 0.5 g/day, and sulfadlazine 2.5 g/day; Eurovet bv, The Netherlands) was administered orally to the recipients from five days before until five days after embryo transfer. The recipients were checked daily for

estrus symptoms. After transfer on Day 7 after hCG, the recipients were checked by ultrasonography for embryonic fluid in the uterus and corpora lutea on the ovaries once weekly from three weeks after ovulation onwards. Thirty days after transfer (Day 37 after hCG) the pregnant recipients were slaughtered to evaluate the survival and development of the transferred embryos. The number and the weight and length of the normal fetuses was determined and the number of embryonic or fetal remnants (degenerating fetuses and remnants of embryonic or placental tissue) was counted.

Statistical analysis

Data are expressed as mean ± SD. Pregnancy rates and number of CL at Day 30 after transfer (Day 37 after hCG) were analyzed by Chi-square or Fisher's Exact Test (depending on number of observations) using the FREQ procedure (SAS 1990). Embryonic (blastocyst) recovery and embryonic development (as average per pool) were analyzed by the GLM procedure (SAS 1990) by eCG dosage (1000 vs 1500 iu), ovulation response (≤25 CL vs >25 CL) and their interaction (if significant). The same procedures were used for number of fetuses or implantations and fetal development. Embryonic development per litter was post-priory classified to below or above overall average development, and likewise related to pregnancy results. The relation between diameter and number of cells of individual embryos was tested with the REG procedure (SAS 1990).

RESULTS

The time of ovulation of the sows was categorized in the classes <36 h, 36 to 42 h, 43 to 48 h and >48 h after hCG. The percentage of recipients (800 iu eCG; n = 48) per class was 0, 40, 56 and 4% respectively. For the 1000 iu eCG donors (n = 50) the percentage was 0, 38, 57 and 4% and for the 1500 iu eCG donors (n = 38) 8, 33, 51 and 8%.

Of the 80 donor sows used, the overall ovulation rate was significantly lower in the 1000 iu eCG donors compared to the 1500 iu eCG donors (28.5±11.7 CL and 45.7±20.3 CL respectively; P<0.0001). Twenty-four of the 48 (50%) 1000 iu eCG donors had a normal ovulation rate (≤25 CL), while in the 32 1500 iu eCG donors only four (13%) sows had a normal ovulation rate (P<0.001; see Table 1). The average embryo recovery rate of 82.9±16.9% and the percentage transferable embryos (expanded blastocysts) was 56.2±31.4%, these were not affected by eCG dosage or ovulation response (P=0.31 and P=0.96, respectively).

Table 1. Number of sows per treatment group and average (± SD) ovulation rate, embryo recovery rate and percentage expanded blastocysts (exp. blast.) on Day 7 after hCG.

eCG dosage	1000 iu eCG			1500 iu eCG		
Ovulation rate	≤ 25 CL	> 25 CL	Total	≤ 25 CL	> 25 CL	Total
Donors (n) [a]	24	24	48	4	28	32
CL/sow [b]	19.2 ± 4.1	37.9 ± 8.9	28.5 ± 11.7	22.0 ± 3.5	49.0 ± 19.4	45.7 ± 20.3
Embryos/sow [b]	16.3 ± 3.9	31.0 ± 9.7	23.6 ± 10.4	18.3 ± 3.6	39.7 ± 17.6	37.0 ± 17.9
Recovery (%)	88 ± 21	81 ± 15	84 ± 18	83 ± 6	80 ± 15	80 ± 14
Exp. blast. (%)	50 ± 39	63 ± 23	56 ± 32	80 ± 12	53 ± 31	56 ± 30

[a] P<0.001 for eCG dosage

[b] P<0.001 for eCG dosage and ovulation response

For transfer and cell counts 989 expanded blastocysts were retrieved and pooled in 20 groups. For the 1000 iu eCG dosage 12 pools were available (≤25 CL: 6 pools of 1 to 5 donors with 17 to 52 embryos per pool; >25 CL: 6 pools of 2 to 5 donors with 15 to 121 embryos per pool). For the 1500 iu eCG dosage 8 pools were available (≤25 CL: 3 pools of 1 to 2 donors with 10 to 34 embryos per pool; >25 CL: 5 pools of 2 to 7 donors with 70 to 190 embryos per pool). The average diameter of the blastocysts per pool was not affected by eCG dosage or ovulation rate (P=0.45 and P=0.99 respectively). The average number of cells of the blastocysts per pool was significantly affected by the eCG dosage (84.3±15.3 and 70.2±1.9 cells, P=0.02; for 1000 and 1500 iu eCG, respectively) but not by the ovulation response (P>0.05; see Table 2). The relation between diameter (y) and number of cells (x_1) or cell cycles (^{2}log cell count; x_2) for individual embryos was $y = 113.7 + 0.62x_1$ (r^2=0.58; P≤0.001) or $y = -18.8 + 29.2x_2$ (r^2=0.48; P≤0.001).

Table 2. Number and average development (diameter of all embryos and nuclei count of sampled embryos; average per pooled group) of the expanded blastocysts according to the eCG dosage and ovulation response of the donor sows.

eCG dosage	1000 iu eCG			1500 iu eCG		
Ovulation rate	≤ 25 CL	> 25 CL	Total	≤ 25 CL	> 25 CL	Total
No. of groups	6	6	12	3	5	8
Diameter (μm)	162 ± 17	163 ± 8	162 ± 12	159 ± 15	157 ± 9	158 ± 11
(No. of embryos)	(142)	(385)	(527)	(54)	(408)	(462)
Nuclei count [a]	83 ± 21	85 ± 8	84 ± 15	71 ± 3	70 ± 2	70 ± 2
Cell cycles* [a]	6.3 ± 0.3	6.4 ± 0.1	6.3 ± 0.2	6.1 ± 0.0	6.0 ± 0.1	6.1 ± 0.1
(No. of embryos)	(55)	(56)	(111)	(25)	(49)	(74)

* ^{2} log nuclei count

[a] P < 0.05 for eCG dosage

Twenty-seven transfers were performed. Sixteen (59%) animals were pregnant at Day 30 after transfer, 7 (26%) animals showed an extended estrous cycle of >25 days, while 4 (15%) returned to estrus within 24 days after hCG (see Table 3). The number of pregnant recipients was 10/14 (71%) with embryos from the 1000 iu eCG donors and 6/13 (46%) with embryos from the 1500 iu eCG donors (P=0.17). The four transfers with embryos from the normal ovulating donors (three from 1000 iu eCG donors and one from 1500 iu eCG donors) resulted in four pregnancies (100%) compared to 12 pregnancies of the 23 transfers (52%) with embryos from the superovulating donors (P=0.10).

The transferred litters were categorized (post priory) by the diameter of the transferred embryos (smaller or larger then the average of 158 μm). The litters with on average smaller embryos resulted in a pregnancy rate of 43% in comparison to 77% for the litters with larger embryos (P=0.07; see Table 4 and Figure 1A). Also the numbers of implantations and fetuses of 10.8±5.2 and 8.8±3.4 for the litters with smaller blastocysts tend to differ from the 14.9±3.9 and 12.1±2.9 implantations and fetuses for the litters with larger blastocysts (P=0.09 and P=0.06, respectively).

Categorizing the transferred litters by the average number of cells per embryo (estimated from the sampled embryos from the same pool of embryos) had no significant effect on pregnancy rate or number of implantations or fetuses (P>0.10; see Figure 1B). Also the coefficient of variation in diameter or in number of cells (estimated from the sampled embryos from the same pool of embryos) is not related to pregnancy rate or number of implantations or fetuses (P>0.10; data not shown).

Table 3. Number of transfers, pregnancy rate and subsequent embryonic survival on Day 37 after hCG for embryos from 1000 and 1500 iu eCG donors.

eCG dosage	1000 iu	1500 iu	Total
Recipients			
used (n)	14	13	27
return to estrus within 24 days (n (%))	1 (7%)	3 (23%)	4 (15%)
estrus interval ≥ 24 days (n (%))	3 (21%)	4 (31%)	7 (26%)
Pregnant (n (%)	10 (71%)	6 (46%)	16 (59%)
Pregnant recipients			
implantations	12.9±3.0	14.2±7.0	13.4±4.7
normal fetuses	11.1±2.7	10.5±4.6	10.9±3.4
fetal remnants [a]	1.8±1.6	3.7±3.0	2.5±2.3
Fetal weight *	4.1±0.8	4.5±0.5	4.3±0.7
Fetal length *	3.9±0.3	3.9±0.1	3.9±0.2

*excluding two pregnant recipients, slaughtered at Day 45

[a] P=0.01

Table 4. Number of transfers, pregnancy rate and subsequent survival and development (average per recipient) on Day 30 after transfer of litters with an average embryonic development below or above average diameter at the time of transfer.

Size of the embryos	Small	Large	Total
Average diameter (μm) of transferred blastocysts per litter	145–158 (151±4)	159 – 181 (167±8)	145 – 181 (159±10)
Recipients			
Used (n)	14	13	27
Return to estrus within 24 days (n (%))	3 (21%)	1 (8%)	4 (15%)
Estrus interval ≥ 24 days (n (%))	5 (36%)	2 (15%)	7 (26%)
Pregnant (n (%)) at Day 30 after transfer [a]	6 (43%)	10 (77%)	16 (59%)
Pregnant Recipients			
Implantations [b]	10.8±5.2	14.9±3.9	13.4±4.7
Normal fetuses [c]	8.8±3.4	12.1±2.9	10.9±3.4
Fetal remnants	2.0±2.5	2.8±2.3	2.5±2.3
Fetal weight*	4.4 ± 0.4	4.1 ± 0.8	4.3 ± 0.7
Fetal length*	4.0 ± 0.1	3.9 ± 0.3	4.0 ± 0.2

* excluding two pregnant recipients, which were slaughtered at Day 45

[a] P = 0.07

[b] P = 0.09

[c] P = 0.06

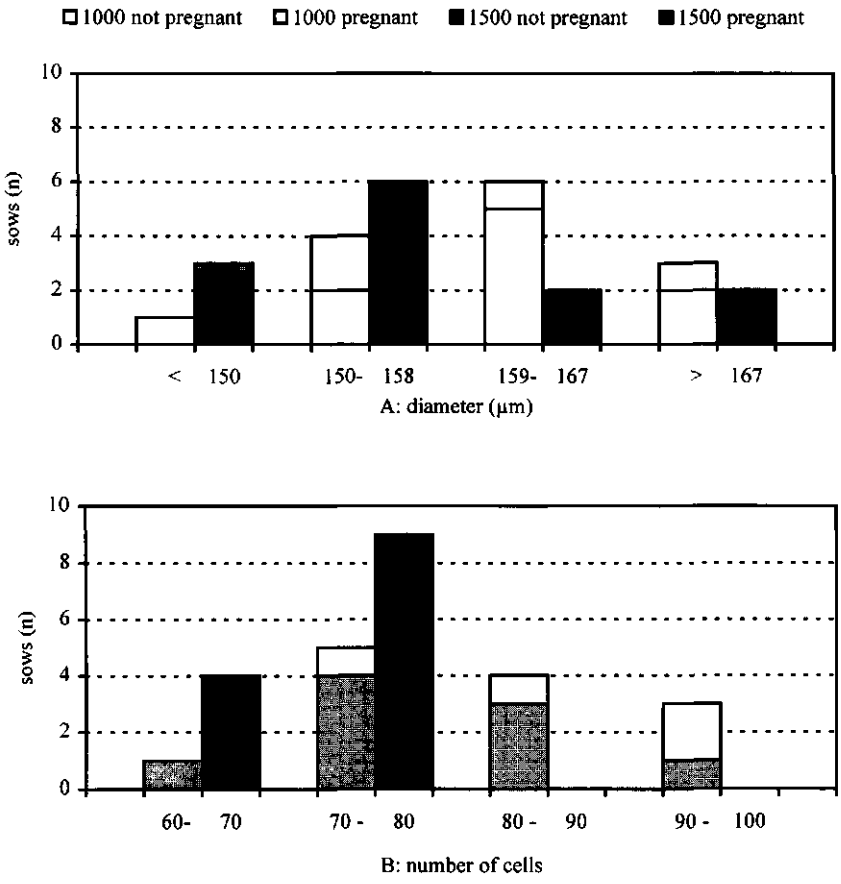

Figure 1 Frequency of sows pregnant or not pregnant after transfer of embryos classified to average diameter (A) or number of cells (B) per litter from donors treated with 1000 or 15000 iu eCG

DISCUSSION

The results of this experiment show that a higher dosage of eCG results in an increase in number of ovulations and of transferable Day 5 embryos, but the recovery and developmental stage expressed as percentage expanded blastocysts are not affected. However, the number of cells of the expanded blastocysts is significantly impaired with the higher eCG dosage (84.3±15.3 and 70.2±1.9 for 1000 and 1500 iu eCG respectively, P=0.02). This effect does not seem to be due to the ovulation rate of the donors, since the development of the embryos (diameter, number of cells or cell cycles) from donors with a normal ovulation rate (≤25 CL) was similar to the embryos from superovulated donors (>25 CL). Therefore the effect of eCG dosage on embryonic development is probably not due to the number of follicles selected. Since time of ovulation was similar in both eCG groups, this dosage effect on embryonic development was also not due to differences in embryonic age. Previous research (Kirkwood et al. 1994, 1995), using 750 iu eCG in gilts, showed no positive effect of eCG neutralization after ovulation on reproductive performance, and Brüssow (1992), using 1500 iu eCG in gilts, even showed a negative effect of eCG neutralization on Day 5 embryonic development. This might indicate that during early embryonic development no serious negative effects of circulating eCG exist. Therefore the negative effect of the higher eCG dosage on embryonic development is probably due to other aspects like oocyte quality, fertilization or early embryonic development. The negative effects of exogenous gonadotropins on oocytes and embryo quality in cattle are well known (Greve et al. 1995), and also in pigs it is known that exogenous gonadotropins have effects on variation in follicular development with consequences for oocyte maturation and hormonal processes (Hunter and Wiesak 1990). Also the number of good quality embryos have been found to be less with increasing dosage of gonadotropins (Schlieper and Holtz 1991), although in our study no effect was found. Effects of eCG dosage on embryonic development may therefore be due to a number of factors like oocyte quality and hormonal imbalances causing disturbances in the processes of fertilization and early embryonic development.

The effect of eCG dosage on embryonic development also seems to be reflected in pregnancy rates after transfer (1000 iu eCG: 71%; 1500 iu eCG: 46%; P=0.17). Although not significant, the difference in pregnancy rate might indicate that embryonic development on the moment of transfer affects subsequent development. The significantly higher number of fetal remnants with embryos from the 1500 iu eCG donors (1.8±1.6, 1000 iu eCG and 3.7±3.0, 1500 iu eCG; P≤0.05) is probably related to the (non significantly) higher and more

variable number of implantations with the 1500 iu eCG embryos (14.2±7.0) in comparison with the 1000 iu eCG embryos (12.9±3.0). High numbers of implantations might cause crowding and thereby mortality after implantation in a number of sows.

Categorizing the litters to lower or higher average blastocyst diameter than the overall average diameter, shows that litters with a larger average diameter tend to have a higher pregnancy rate and more implantations and fetuses. This might also explain the lower pregnancy rates with the high eCG dosage since in these transfers most litters showed a lower than average diameter, resulting in a lower number of pregnancies (Figure 1A). As can be seen in Figure 1A, the pregnancy rates predominantly depend on the diameter of the transferred blastocysts and not on the eCG dosage. Although the average blastocyst diameter of the pools was not affected by eCG dosage, the higher eCG dosage resulted in less available litters (= number of available embryos) in the pools with a higher average diameter. A similar division in number of cells, as estimated from sampled embryos from the same pool of embryos as the transferred litters, does not result in differences in pregnancy rate as shown in Figure 1B, despite the lack of litters with high cell numbers in the 1500 iu eCG dosage group. Although diameter is related to the number of cells (r^2=0.58) the two parameters describe different aspects of blastocyst development. The number of cells represent the rate of development (number of cell cycles after ovulation). The diameter however, reflects morphological development and blastocoel formation on the time of observation. These parameters might represent the embryonic functioning at the time of observation or transfer. The blastocoel formation is among others determined by the quality of the tight junctions between the trophoblastic blastomeres and the functioning of the Na/K-ATPase (Watson 1992). Therefore embryonic diameter might be a valuable parameter, reflecting embryonic functioning.

A high within litter diversity is generally considered to be related with embryonic mortality (Van der Lende et al. 1993). However, in this study no relation of within litter variation based on diameter of expanded blastocysts with pregnancy rate or embryonic survival could be found. A cause might be that in our study only expanded blastocysts are transferred.

In summary, the results of this experiment indicate that ovulation rate and number of transferable embryos increase with a higher eCG dosage. The quality of these embryos (expanded blastocysts) in terms of average number of cells but not average diameter is decreased with a higher eCG dosage and these effects are not due to ovulation rate. The survival of the embryos after non-surgical transfer seems to be related to the average diameter of the blastocysts in the transferred litter. Since the diameter is also related to the number of cells, these results indicate that an easy assessable parameter like diameter of the blastocyst gives valuable information about the quality of the embryo and about survival chances after non-surgical transfer.

ACKNOWLEDGEMENTS

This research on embryo transfer has been supported by: Dalland bv, Dumeco Breeding, Dutch Association of AI Centres for Pigs, Dutch Herdbook bv, Euribrid bv and Product Board for Livestock, Meat and Eggs. We acknowledge dr. N.M. Soede and dr. T. van der Lende for their critical review of the manuscript.

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Chapter 6

CHARACTERISTICS OF DONOR AND RECIPIENT SOWS AND TRANSFER PROCEDURE AFFECTING THE RESULTS OF NON-SURGICAL TRANSFERS OF DAY 5 EXPANDED BLASTOCYSTS.

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ABSTRACT:

The aim of this study was to identify factors, other than embryo quality, which might affect the results of non-surgical embryo transfer. In this study data from two different experiments using multiparous sows were combined. Characteristics of donors were related to embryo yield and characteristics of recipients and the execution of the transfer procedure were related to subsequent transfer results. Only transfers with 28 to 32 expanded blastocysts to synchronous recipients were analysed. After synchronisation of the luteal phase by altrenogest (Exp. 1) or induced luteal regression with cloprostenol (Exp. 2), follicular development was stimulated by application of eCG (for donors: 1000 iu (Exp. 1 and Exp. 2A) or 1500 iu (Exp. 2B); for recipients: 1000 iu (Exp. 1) or 800 iu (Exp. 2)). After 72 h, ovulation was induced with 750 iu hCG in all animals. The number of transferable embryos (expanded blastocysts at 120 h after ovulation) per donor was 14.5±1.4 (Exp. 1), 14.3±1.5 (Exp. 2A) and 20.4±2.7 (Exp. 2B; $P<0.05$). The donors of parity 5 to 8 had a higher number of transferable embryos (18.9±1.4) than donors of parity 1 to 4 (13.1±1.7; $P<0.05$), with an intermediate number of transferable embryos (15.2±2.1) for donors of parity 9 to 16. From donors with a high litter size (≥14 piglets born) preceding embryo collection, more (21.1±2.1) transferable embryos were collected than from donors with lower litter size preceding embryo collection (14.4±1.1; $P<0.05$). Other characteristics of the donor sows, like average litter size of all litters produced, lactation length preceding embryo collection, weight at slaughter or duration of oestrus preceding embryo collection were not related to the number of transferable embryos. A short duration of oestrus (36-72h) preceding embryo collection was related with a lower fertilisation rate (84.4±2.8%) compared with a long oestrus duration (≥84 h: 94.1±1.9%; $P<0.05$).

After transfer, at Day 35 after ovulation, a pregnancy rate of 60% in Exp. 1 (n=20) and 59% in Exp. 2 (n=27) was achieved ($P>0.05$). None of the recipient characteristics (e.g. previous litter size, lactation length, parity) differed between pregnant and non-pregnant recipients ($P>0.05$). Also no relations between pregnancy rate and scores of the execution of the transfer procedure like reaction of the sow to introduction of the transfer pipette, resistance felt in the cervix, depth of introduction of the pipette were detected ($P>0.05$). The duration of the transfer procedure did not differ for pregnant and non-pregnant recipients (5.0±0.5 and 5.5±0.5 min, respectively; $P>0.05$). The time between embryo collection and transfer was also not different (pregnant: 5.6±0.2 h and non-pregnant: 5.4±0.2 h; $P>0.05$).

It was concluded that some characteristics of the donors affected the yield of transferable embryos, but characteristics of the recipients or aspects of the transfer procedure did not affect the results of the non-surgical transfers.

Keywords: non-surgical embryo transfer, pregnancy rate, donors, recipients

INTRODUCTION:

For embryo transfer, donors are required that give high numbers of good quality (transferable) embryos and recipients are required that give high pregnancy rates and large litter sizes. It might be expected that sows with good reproduction characteristics, like high litter sizes, will be the most suitable animals for these purposes. Furthermore, it is important to gain knowledge about parameters describing the transfer procedure that might affect the results in terms of pregnancy rate or litter size. Aspects like difficulties during introduction of the instruments into the sows have been described to decrease pregnancy rates for specific procedures (Galvin et al. 1994, Yonemura et al. 1996, Li et al. 1996). In our previous studies no obvious differences in the results related to our transfer procedure were detected (unpublished results).

This study aims to identify characteristics of donors that are related to embryo yield and characteristics of recipients that are related to pregnancy rates and litter sizes. Furthermore parameters describing characteristics of the execution of the transfer procedure are related to the transfer results.

MATERIAL AND METHODS:

This study is based on a pilot experiment with unpublished results (Exp. 1) and a previously published, comparable experiment (Exp. 2; Hazeleger et al. 1999).

Animals:

Twenty batches of 10 to 20 commercial crossbred (mainly Landrace x Yorkshire) sows (n=304; parity 1 to 16), arrived on the experimental station on the day of weaning (approx. 3 to 4 weeks after farrowing). In Exp. 1 the first or second post-lactation oestrus cycle of these sows was synchronised by treating them (orally) with 20 mg Altrenogest (Regumate® Pig, Hoechst Roussel Vet NV, Belgium) per sow from approximately 12 days after onset of oestrus for a period of 7 days. In Exp. 2, the cycle of the sows was synchronised by luteolysis induction on approximately 15 days after onset of oestrus by means of two injections (i.m.; interval 8 h) of 500 µg cloprostenol (Estrumate®; Malinckrodt Veterinary bv, The Netherlands). The day after last altrenogest treatment (Exp. 1) or the day after luteolysis induction (Exp. 2), the sows were treated with eCG (Folligonan®; Intervet bv, The Netherlands; 1000 iu per donor in Exp. 1 and Exp. 2A or 1500 iu in Exp. 2B; 1000 iu per recipient in Exp. 1 or 800 iu per recipient in Exp. 2). Ovulation was induced three days (72h) later with 750 IU hCG (Chorulon®, Intervet bv, The Netherlands). Transrectal ultrasonography (Scanner 150S, Pie Medical b.v., Maastricht, The

Netherlands) was performed at 36 and 48 h after hCG to monitor the time of ovulation. Only sows ovulating between 36 and 48 h after hCG were used in these experiments. Oestrus was checked by the back pressure test in front of a boar twice daily. Donors were artificially inseminated on 36 and 42 h after hCG with a commercial dose of 80 mL ($2x10^9$ sperm cells) semen.

The donors were weighed and slaughtered for embryo collection at 162 h after hCG (approximately 120 h after ovulation) as previously described (Hazeleger et al. 1999). The number of corpora lutea (CL) on the ovaries and the number of collected eggs/embryos were counted and development of the eggs/embryos was scored and classified in unfertilised, morulae, blastocyst, expanded blastocyst and hatched blastocyst. In Exp. 1, the embryos to be transferred (all non-hatched blastocyst stages) were gathered from one donor and additional embryos with a similar development from other donors were added to create litters of approximately 30 (range 29 to 32) embryos. This resulted in 11 litters with blastocysts and expanded blastocysts together and in 20 litters with expanded blastocysts only. In Exp. 2, only expanded blastocysts were collected, pooled per batch (1 to 6 donors) and divided in 27 litters of 28 to 30 embryos to be transferred.

Transfer procedure:

The embryos were transferred as previously described (Hazeleger et al. 1999). The transfer procedure consisted of a careful passage of the instrument through the cervical folds into the uterine body. The embryo catheter, containing the embryos in the tip, was passed through the instrument and the embryos were placed in the uterine body with <0.1 mL Dulbecco's PBS (Sigma, USA) with 10% heat inactivated lamb serum (Gibco, UK). The recipients were not sedated during transfer.

After transfer, the recipients were checked for oestrus symptoms twice daily. From three weeks after ovulation onwards, the recipients were checked by ultrasonography for embryonic/foetal fluid and corpora lutea (CL) once weekly. On Day 35 after ovulation (30 days after transfer) the pregnant recipients were slaughtered to evaluate the survival of the transferred embryos. The number of implantations (including placental remnants and degenerating foetuses) and number of foetuses with a normal appearance were counted. The recipients returning to oestrus were slaughtered within a few days after oestrus to detect possible anatomical disorders which might explain the return to oestrus.

Statistical analysis:

The analysed sow characteristics of donors and recipients were: parity, weight at slaughter, lactation length (preceding embryo transfer), litter size (preceding embryo transfer), average litter size (of all litters produced) and oestrous duration (preceding embryo transfer).

The characteristics of the procedure of embryo transfer which were related to transfer results were: the time between embryo collection and transfer (h), the duration of the transfer procedure (min) and other procedural characteristics, subjectively scored in 5 classes:

- Reaction of sow = reaction of the sow during insertion of the pipette (1= no reaction; 5= maximal reaction, struggling),
- Resistance in cervix = resistance felt during movement of the pipette through the cervix (1= almost no resistance; 5= severe resistance),
- Depth in uterus = subjective score of the introduction depth of the instrument in the uterus (1= just behind the cervix; 5= approximately 10 cm behind the cervix),
- Tissue damage = whether or not the instrument showed signs of blood after the transfer procedure (1= clean; 5= clear signs of blood).

Analysis of donors: Percentage fertilised eggs (morulae and blastocyst stages) was expressed as percentage of the total number of eggs/embryos collected. Percentage transferable embryos (expanded blastocysts) was expressed as percentage of the number of fertilised eggs.

Number of corpora lutea (CL) and number or percentage of transferable embryos were related with the characteristics of the donors, classified in three (parity, weight and lactation length) or five (litter size and oestrus duration) classes of approximately equal size. Characteristics of the donors were analysed separately, taking into account significant effects of experiment and interaction between experiment and sow characteristics (Proc GLM; SAS 1990).

The relation between percentage fertilised eggs and sow characteristics was analysed by non-parametric procedures (Kruskal-Wallis test; Proc NPAR; SAS 1990). The relation with oestrus duration (classified in classes of approximately equal size) was tested by X-square analysis (Proc FREQ; SAS 1990).

Additionally a group of nine sows showing no oestrus symptoms, but ovulating at the expected time, were inseminated and slaughtered for embryo collection. Their number of CL, number and percentage transferable embryos were compared with the results of the other sows (that did show oestrus symptoms and ovulation at the expected time), by one-way analysis of variance (Proc GLM; SAS 1990) and fertilisation rate was analysed by non-parametric procedures (Kruskal-Wallis test; Proc NPAR; SAS 1990).

Analysis of recipients and transfer procedure: Differences between pregnant and non-pregnant recipients in the sow characteristics, duration of the transfer procedure and time period between embryo collection and transfer were tested separately and (if significant) in combination with Experiment (1, 2A and 2B) and their interactions (SAS: Proc GLM). Within the pregnant recipients the number of foetuses and implantations were similarly analysed. The classified characteristics of the execution of the transfer procedure were related with pregnancy rate, number of foetuses and number of implantations (both classified in three classes of equal size by X-square or Fisher's Exact (SAS: Proc FREQ).

All means are expressed as mean ± SEM.

RESULTS

Animals:

In total, 304 animals were synchronised. Forty-six (15.1%) animals showed no obvious oestrus symptoms around the time of hCG application and were not used. Ovulation was checked in 248 sows showing oestrous symptoms of which 10 sows (4.0%) ovulated too early (< 36 h after hCG) and 14 sows (5.6%) ovulated too late (> 48 h after hCG), while 224 animals (90.3%) ovulated within the expected range of 36 to 48 h after hCG, and were subsequently used for analysis. Because sometimes not enough donors or recipients were available at the right time, 145 donors and 47 recipients (receiving expanded blastocysts only) were analysed. The average (range) values of the sow characteristics of these animals were:

- Parity 6.8 ± 0.2 (1 – 16)
- Weight at slaughter (kg) 196.0 ± 1.9 (131 – 280)
- Lactation length (d) 25.1 ±0.4 (4 – 39)
- Previous litter size (piglets) 11.1 ± 0.3 (3 – 21)
- Average litter size (piglets) 11.2 ± 0.2 (6.5 – 17.0)
- Oestrus duration (h) 73.1 ± 1.2 (36 – 108)

Donor characteristics and embryo yield:

The average number of CL, number of recovered eggs and number of transferable embryos are affected by experiment (Table 1). In Exp.2B (1500 iu eCG) the number of CL (45.8±3.7) is higher than in the 1000 iu eCG groups (Exp. 1: 29.4±1.5 and Exp.2A: 28.8±1.7, respectively; $P<0.0001$; Table 1). The number of transferable embryos in Exp. 2B (20.4±2.7) differed correspondingly from Exp. 1 and 2A (14.5±1.4 and 14.3±1.5, respectively; $P<0.05$; Table 1). Percentages recovery, fertilisation and transferable embryos were not affected by experimental group.

Table 1. Average ovulation rate (CL) and number and percentage recovered eggs, fertilisation rate, and number and percentage transferable embryos as found in the different experiments.

	Exp. 1	Exp. 2A	Exp. 2B
Nr. of obs.	67	47	31
CL	29.4 ± 1.5^{a}	28.8 ± 1.7^{a}	45.8 ± 3.7^{b}
Nr. of eggs	23.8 ± 1.4^{a}	23.7 ± 1.5^{a}	37.2 ± 3.3^{b}
Recovery (%)	81.0 ± 2.0	83.4 ± 2.5	80.8 ± 2.6
% Fertilised	88.6 ± 2.7	90.5 ± 2.8	84.1 ± 5.1
Nr. Transferable embryos	14.5 ± 1.4^{a}	14.3 ± 1.5^{a}	20.4 ± 2.7^{b}
% Transferable embryos	62.0 ± 4.4	63.0 ± 4.6	70.8 ± 4.4

^{a,b} different superscripts in a row differ significantly, $P<0.05$

No significant interactions between donor characteristics and experimental group were detected for the number of CL, the number of transferable embryos or percentage transferable embryos. The donor characteristics parity and previous litter size were related with the number (corrected for experimental group) and percentage of transferable embryos ($P<0.05$; Table 2).

Table 2. Average number of ovulations (CL) and number and percentage transferable embryos at 120 h after ovulation for synchronised sows (1000 (n=114) or 1500 (n=31) iu eCG and 750 iu hCG), depending on parity and last litter size.

	n	CL	Transferable (n)	Transferable (%)
Parity				
1-4	47	30.4 ± 2.4^{a}	13.1 ± 1.7^{a}	62.6 ± 5.1^{ab}
5-8	52	32.8 ± 1.8^{ab}	18.9 ± 1.4^{b}	74.4 ± 3.2^{a}
9-16	43	35.9 ± 2.7^{b}	15.2 ± 2.1^{ab}	55.5 ± 5.3^{b}
Last litter size (piglets born)				
≤ 7	18	31.9 ± 4.2	12.1 ± 2.5^{a}	47.8 ± 8.3^{a}
8-9	30	32.9 ± 3.3	16.5 ± 2.8^{ab}	65.7 ± 5.7^{abc}
10-11	29	29.4 ± 2.5	13.3 ± 2.3^{a}	54.7 ± 6.8^{a}
12-13	17	36.8 ± 4.3	15.6 ± 2.1^{ab}	68.9 ± 6.6^{bc}
≥ 14	28	35.3 ± 2.7	21.1 ± 2.1^{b}	81.7 ± 4.6^{c}

^{a,b,c} different superscripts differ significantly $P<0.05$.

The donors with parity 5 to 8 had a higher number of transferable embryos (18.9±1.4) than parity 1 to 4 (13.1±1.7, P<0.05), while parity 9 to 16 (15.2±2.1) had an intermediate number. The percentage transferable embryos was higher for parity 5 to 8 (74.4±3.2%) in comparison with parity 9 to 16 (55.5±5.3%, P<0.05), while parity 1 to 4 had an intermediate percentage (62.6±5.1%). If the previous litter size was ≥ 14 piglets born, the number and percentage of transferable embryos was higher (21.1±2.1 embryos, 81.7±4.6%) in comparison with the average of all other classes of sows with a lower (< 14) litter size (14.4±1.1 transferable embryos, 60.1±3.0%; P<0.05). The other parameters (i.e. lactation length, average size of all previous litters, oestrus duration and weight at slaughter) were not related with the number or percentage of transferable embryos (P>0.05).

Fertilisation rate was positively related to oestrus duration (P<0.05; Figure 1). Sows with a long oestrus duration (84 h or more) had a fertilisation rate of 94.1±1.9%, while sows with an oestrus duration of 36 to 72 h had a fertilisation rate of 84.4±2.8% (P<0.05), although no significant differences in number or percentage transferable embryos were found. In addition to these data, nine donors which showed no obvious oestrus symptoms, were inseminated according to the time of ovulation and ovulated within the normal time (36 to 48 h after hCG) were also slaughtered. They had a low number of transferable embryos (5.6±2.5) compared with the average of the other sows (16.0±1.0; P<0.05), due to a lower recovery and fertilisation rate (60.2±13.3% and 70.6±11.1%, respectively) compared with the average of the other sows (81.7±1.3% and 88.3±1.9%, P<0.001 and P<0.05; respectively; Figure 1). Their ovulation rate (29.1±3.7 CL) did not differ from that in the other animals (32.7±1.3 CL; P>0.05).

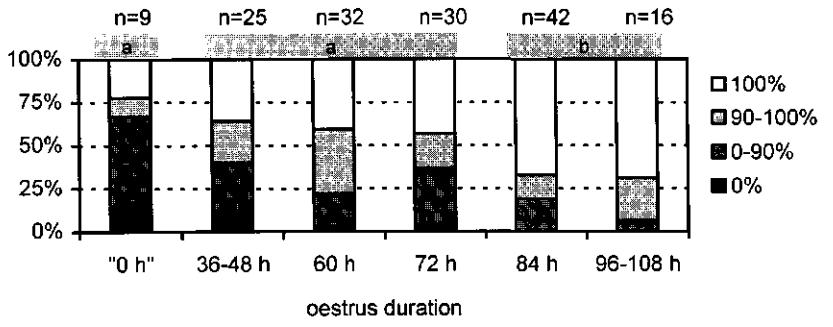

Figure 1. Donors classified in fertilization classes (0%, 0-90%, 90-100%, 100%) in relation to oestrus duration. "0 h"-group is added to illustrate the results of this group in comparison to the other groups. (a vs b, P< 0.05)

Recipient characteristics and transfer results:

In Exp. 1, 31 transfers were performed. Eleven transfers were done with litters of 30 embryos containing non-expanded blastocysts and expanded blastocysts together. These transfers resulted in a pregnancy rate of 27% (3/11) and 20 transfers were done with litters of 30 expanded blastocysts only, resulting in a pregnancy rate of 60% (12/20; P=0.08; Table 3). The results of the latter group (only expanded blastocysts transferred) are similar to the results of Exp. 2, in which also only expanded blastocysts were transferred, resulting in a pregnancy rate of 59% (16/27; P>0.05, Table 3). The data from transfers with only expanded blastocysts of Exp. 1 (n=20) and Exp. 2 (n=27) are used for further analysis. Within these data no effects of experiment on transfer results were found.

Table 3. Pregnancy rate and average number of foetuses and implantations at Day 35 after ovulation obtained in the two experiments.

	Experiment 1		Experiment 2
	<100% Exp. Bl.[1]	100% Exp. Bl.	100% Exp. Bl
Pregnancy rate (%)	3/11 (27%)	12/20 (60%)	16/27 (59%)
Nr. of fetuses	8.0 ± 1.7	9.2 ± 0.9	10.9 ± 0.8
Nr. of implantations	9.3 ± 3.0	10.7 ± 1.3	13.4 ± 1.2

[1] Exp. Bl. = Expanded Blastocysts

No differences could be detected between pregnant and non pregnant sows in sow factors like parity, average litter size, last litter size, lactation length, duration of oestrus or weight at slaughter (Table 4). Post mortem studies revealed no anatomical disorders in the non-pregnant recipients. Within the group of pregnant recipients no relation could be found between the sow characteristics and the number of foetuses or implantations (data not shown; P>0.05).

Transfer procedure characteristics and transfer results:

The average duration of the transfer procedure was 5.2±0.3 (2–15) min and no differences between non-pregnant and pregnant recipients were found (P>0.05; Table 4). The average time between embryo collection and transfer was 5.5±0.1 (3–8) h and was not different between non-pregnant recipients and pregnant recipients (P>0.05; Table 4). These parameters were also not related to the number of foetuses or implantations within the pregnant recipients (data not shown; P>0.05).

No relations of pregnancy rate with scores of the execution of the transfer procedure like the reaction of the sow, resistance of the cervix, depth of introduction or tissue damage could be detected (P>0.05; Table 5). These parameters were not related to the number of foetuses or implantations within the pregnant recipients either (data not shown; P>0.05).

Table 4. Average values of sow characteristics, duration of the transfer procedure and embryo storage for pregnant and non-pregnant recipients.

	Not pregnant	Pregnant	P-value
Nr of transfers	19	28	
Sow characteristics:			
Parity	7.4 ± 0.7	7.6 ± 0.5	0.82
Average litter size	12.1 ± 0.4	11.8 ± 0.5	0.62
Last litter size	11.7 ± 0.6	11.8 ± 0.5	0.97
Last lactation length	26.0 ± 5.1	25.6 ± 3.8	0.72
Estrus duration	77.3 ± 3.5	77.8 ± 2.7	0.92
Weight at slaughter	206 ± 5	202 ± 4	0.50
Transfer procedure characteristics:			
Duration transfer (min)	5.5 ± 0.5	5.0 ± 0.5	0.51
Duration embryo storage (h)	5.4 ± 0.2	5.6 ± 0.2	0.37

Table 5. Relations between scores of the execution of the transfer procedure with pregnancy rate.

Score[1)]	1	2	3	4	5
Reaction of sow	59%	25%	75%	100%	-
	n=37	n=4	n=4	n=2	n=0
Resistance in cervix	83%	25%	63%	67%	33%
	n=6	n=10	n=19	n=9	n=3
Depth in uterus	100%	63%	57%	40%	100%
	n=2	n=16	n=23	n=5	n=1
Tissue damage	61%	43%	100%	100%	-
	n=38	n=7	n=1	n=1	n=0

[1)] 1 = minimum score , 5 = maximum score.
n = number of transfers.

DISCUSSION

Donor characteristics and embryo yield

In donor sows, parity and litter size preceding embryo collection were related with the number and percentage of transferable embryos (expanded blastocysts at 120 h after ovulation, induced at the second oestrus after weaning). It is known that the largest litter sizes are realised from parity 4 to 7 (Vesseur et al. 1994), what corresponds with the number of transferable embryos in our study. Successive litter sizes are reported to be significantly correlated (Hughes 1998). The relation of litter size preceding embryo collection with the quality of the embryos of a sow indicates that embryonic development represents certain important aspects of the reproductive capacity of a sow, even in the case when embryos are produced by artificial regulation of follicular development and ovulation.

Although embryonic development is only one of the many factors determining litter size, it implies that the embryonic developmental stage is important for survival in normal pregnancies as well as after transfer. The latter is found in Exp. 1, in which transfer of expanded blastocysts (without less developed blastocysts) resulted in the highest pregnancy rates. Several other studies show the importance of well developed embryos for maximal embryo survival after embryo transfer (Pope et al. 1982, Wilmut et al. 1985, Hazeleger and Kemp 1994). The relation between the quality (% expanded blastocysts at 120 h after ovulation) and number of transferable embryos and the sow characteristics parity and last litter size preceding embryo collection, offers criteria for selection of multiparous donor sows for optimal embryo yields.

The finding that in the donor sows a short oestrus duration was related to poor fertilisation results is difficult to explain because all sows are inseminated twice, regardless of the time of first oestrus symptoms, just before and around the time of ovulation (36 and 42 h after hCG and time of ovulation checked by ultrasound) aimed at optimal fertilisation results. Poor fertilisation results after ovulation induction are also reported in gilts, even with low dosages of eCG and hCG (Holtz and Schliepper 1991). However, they did not present results about oestrus expression and also the time of ovulation was not checked and deviations from the expected time of ovulation might have caused suboptimal fertilisation. The short oestrus periods with reduced fertilisation in our study might reflect suboptimal hormonal processes, affecting the fertilisation process. Indirect evidence indicated that short oestrus duration is not related with poor fertilisation under natural conditions (Kemp and Soede 1996). Therefore, these suboptimal hormonal processes might be caused by our synchronisation treatments. However, between low and high dose of eCG in Exp. 2, no differences in fertilisation rate or

oestrus duration (1000 iu: 76±2.5 h; 1500 iu: 72±2.4 h; P>0.05) were detected. Since hCG was given at a fixed time (72 h) after eCG administration, regardless of the time of first oestrus symptoms, this injection might have been too early for some sows and therefore the short period of oestrus expression might reflect poor follicular development and a low oestrogen secretion at the time of hCG treatment. This is in agreement with results of Nissen et al. (1995), reporting reduced follicular development and poor oestrus expression in sows treated with hCG 3 days after weaning, although no significant differences in embryonic recovery at Day 8 after ovulation were found. An indication for this might be the positive effect of oestradiol on the sperm binding to oviductal cells as found in in vitro experiments (Suarez et al. 1991). This might indicate that low oestrogen levels, besides their effects on oestrus expression, have a negative effect on sperm reservoirs in the oviduct.

It seems that in sows with a short oestrus period and poor fertilisation rates, the ovulation is induced too early.

Recipient characteristics and transfer results

In total 31 pregnancies were obtained from 58 transfers (53%). After exclusion of 11 transfers with less developed embryos (litters containing non-expanded blastocysts the pregnancy rate was 60%, 28 pregnancies from 47 transfers with expanded blastocysts only, with no differences in results between experiments. The characteristics of the recipients parity, previous litter size, previous lactation length, oestrus duration and body weight were not related to the results of the transfers. Apparently, these general characteristics are not related to the quality of the uterus. It might be that no effect is found because the sows have been cyclic after weaning and the embryos are transferred during the synchronised second oestrus cycle after weaning. Differences in uterine development and secretions between first-oestrus and multi-oestrus gilts have been reported (Murray and Griffo 1976, Schnurrbusch and Erices 1979). Although embryonic survival after transfer differed between first- and second-oestrus donors (63.8 and 84.9%, respectively; P<0.05), no differences in transfer results are found for surgical transfers to first- and third-oestrus recipients (pregnancy rate 67.5 and 60.0%, embryonic survival 76.1 and 78.2%, respectively, P>0.05; Archibong et al. 1992).

The results of our study seem to indicate that the general recipient characteristics measured (last litter size, parity, etc.) do not reflect differences in uterine quality, since no relation with transfer results was found.

Transfer procedure characteristics and transfer results

In this study, characteristics of the procedure of non-surgical transfer were not related to the results of the transfers. Characteristics which might indicate difficulties with the

introduction of the instrument, like the resistance felt in the cervical canal, tissue damage after implementation of the procedure or duration of the transfer procedure are not related to the success rate of the transfers. This is in contradiction with results published by Galvin et al. (1994), Li et al. (1996) and Yonemura et al. (1996). They found poor results when the introduction of the transfer instrument gave problems. The reason might be that Galvin et al. (1994) and Yonemura et al. (1996) were using a procedure which was more similar to an AI procedure. In the case they had more problems with the introduction, the instrument remained somewhere in the cervical canal. The embryos were deposited in the cervix and had to be flushed further into the uterus. This might have caused adherence of the embryos to cervical mucus. In the study of Li et al. (1996) it is not clear, whether the instrument had entered the uterus, when difficulties were observed. In our study the instrument was always introduced into the uterus, thereby preventing these problems. This might explain that no relation was found with difficulties of the transfer procedure.

The storage time of the embryos of three to seven hours at 25^0C, was not related with pregnancy rates. In most other studies the embryos were stored for approximately three to five hours at temperatures ranging from room temperature (Polge and Day 1968, Reichenbach et al. 1993) to 39^0C (Galvin et al. 1994, Li et al. 1996). However, in the study of Yonemura et al. (1996) embryos were even stored up to 20 hours at 37^0C. Also storage periods of up to 36 h at 36.5^0C were reported, resulting in successful pregnancies after surgical transfer (Niemann et al. 1989). Temperatures of 15^0C and lower are reported to be lethal for pig embryos, but temperatures of 20^0C or 37^0C appear to be adequate for storing embryos (Wilmut 1972, Blum and Holtz 1988, Plante et al. 1993). The storage time and temperature of pig embryos seem not very critical within the ranges of approximately 2 to 36 h and 20 to 39^0C, respectively.

In the future, with more practical experience, more knowledge about factors and conditions affecting the transfer results will be gained.

In general it can be concluded that some characteristics of multiparous sows, such as parity and litter size preceding embryo collection, affected the number and percentage transferable embryos in multiparous donor sows. None of such characteristics of the recipients were related to the transfer results after synchronous non-surgical embryo transfer, neither did the characteristics of the procedure of embryo transfer.

AKNOWLEDGEMENTS

We thank Dr. N.M. Soede and Dr. T. van der Lende for their critical review of the manuscript. Research on embryo transfer in our department has been supported, in part, by Dalland BV, Stamboek Pigs, Dumeco Breeding BV, Dutch Association of AI Centres for Pigs, Euribrid BV and Product Board for Livestock, Meat and Eggs.

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GENERAL DISCUSSION

Published in modified form as:
"State of the art in pig embryo transfer"
Theriogenology 51 (1999) 81-90
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GENERAL DISCUSSION

INTRODUCTION

In pigs, embryo transfer has been performed surgically for several decades. For commercial applications it has been used only to a limited extent. This is due mainly to practical implications of surgical procedures like the need for surgical facilities (room and equipment) and anesthesia. The need for extra offspring from a genetic point of view is also low, due to the high fecundity of pigs. However, the increased need for transfer of valuable genetic material around the world with minimal health risks and low costs, has led to stronger motivation to use embryo transfer technologies in pig breeding and production. The successful application in cattle of non-surgical embryo collection and transfer and related techniques (e.g., embryo freezing, ultrasound-guided trans-vaginal ovum pick-up, in vitro embryo production) stimulated the demand to develop similar techniques for pigs. Important for the successful application of such new reproduction techniques is the development of a reliable, efficient technique for embryo transfer, preferably in a non-surgical or minimally invasive way, without the disadvantages of surgical procedures. Likewise, it is to be expected that the availability of non-surgical or endoscopic techniques for pig breeding will contribute substantially to the development and commercial use of other reproductive technologies.

In this paper, state of the art, developments, results and feasibility of novel embryo transfer techniques in pigs will be reviewed. Subsequently, some relevant embryo collection techniques and potential applications of these procedures for the future will also be reviewed.

SURGICAL PROCEDURES

Surgical procedures for transferring embryos into recipients have been available for several decades and research into factors affecting their results has been summarized as early as 1982 (Polge). Surgical procedures for commercial application have only been used to a limited extent (Cameron et al. 1990). These procedures will be briefly described for comparison with recently developed techniques. In principle, all these techniques are performed under general anesthesia, with the genital tract presented through a midventral incision in the caudal abdominal region. Usually gilts are used because they are easy to handle and tolerate anesthesia and surgical procedures better than sows. The embryos are placed in

the oviduct or tip of the uterine horn, depending on the developmental stage of the embryos. They are deposited using a small pipette or thin tubing in a small volume of transfer medium. On average, the pregnancy rate is about 60%, and the litter size is 6.5 piglets, with a range of pregnancy rate from 17% with 2.4 piglets to 100% and an average litter size of nearly 10.8 piglets (Brüssow and König 1988).

ENDOSCOPIC PROCEDURES

Endoscopic procedures were developed more recently (Stein-Stefani and Holtz 1987, Besenfelder et al. 1997). Although they can be defined as surgical techniques, their advantage is that only a few small incisions need to be made for the instruments. However, the endoscopic procedures require anesthesia and other surgical precautions similar to those of the conventional surgical approaches, and are therefore less applicable for use on individual farms. Pregnancy rates have ranged from 14% (2/14) with 8 and 9 piglets (24) to 40% (5/12), with an average of 7 piglets per litter for transfers into the uterus, and 33% (9/27) with an average of 6 piglets per litter for tubal transfers (Besenfelder et al. 1997). Recently, pregnancy rates of 90% for routine application of endoscopic ET (including endoscopic embryo collection) were reported but detailed results were not presented (Besenfelder et al. 1998). Together, these results indicate that such a minimally invasive surgical method of transferring embryos can be readily used.

NON-SURGICAL PROCEDURES

Non-surgical procedures are based on a transcervical approach to collect embryos from and/or introduce them into the uterus. After an initial experiment in 1968 (Polge and Day), these procedures have been developed during the last decade. Polge and Day (1968) transferred pig embryos from slaughtered donors (Days 3 to 5 after insemination) to synchronous anesthetized recipients. Only 1 of the 17 recipients was pregnant on Day 17 after transfer. Three developing embryos and four embryonic remnants were found at slaughter. They concluded that the uterus of a sow that is not in estrus, has a cervix that is difficult to enter and that the cervical or uterine wall could be easily perforated. This might be a major factor for the poor results obtained. Sims and First (1987) performed transfers of Day 7 embryos by flushing the embryos through an AI-spirette into the uterus of mildly sedated recipients. These animals were slaughtered at an unknown stage of pregnancy and, at the time of slaughter, 13 out of 21 transfers had resulted in pregnancies.

Subsequent experiments with non-surgical procedures were reported in detail by 5 different research groups (Reichenbach et al. 1993, Galvin et al. 1994, Li et al. 1996, Yonemura et al. 1996, Chapter 3 and 5; summarized in Table 1), all using different approaches. The three main differences between procedures are 1) use of recipient sedation, 2) type of instrument used (AI-spirette or special design), and 3) the volume of fluid used for transfer.

Table 1. Summary of procedures and results of recently published non-surgical transfer procedures

References	Reichenbach *et al.* (1993)	Galvin *et al.* (1994)	Hazeleger, Kemp [1)] (1994)	Li *et al.* (1996)	Yonemura *et al.* (1996)	Hazeleger *et al.* [2)] (1999)
Sedation	Yes	Yes	No	Yes	No	No
Procedure [a]	AI + SI	AI	SI	AI + SI	AI	SI
Medium (mL)	10-20	10-12	≤ 0.1	<0.3	30 or 50	≤ 0.1
Embryos (n)	25-40	12±0.5	17±2	23±10	18±8	28-30
Stage of embryo	8 cell to hatched blastocyst	4 cell to hatched blastocyst	Morula to blastocyst	4 cell to blastocyst	4 cell to hatched blastocyst	expanded blastocyst
Farrowing rate (%)	5/58 [c] (9%)	10/46 (22%)	7/21 (33%)	5/16 (31%)	16/25 (64%)	16/27 [d] (59%)
Litter size (range)	2, 6, 7	4.3±0.7 (3-6)	6.7±1.6 (4-9)	6.2±3.1 (3-10)	3.1±1.6 (1-7)	10.9±3.4 (3-15)
% Survival [b] (range)	7.4, 20.0, 21.9	35±13 (21-63)	38±8 (22-47)	31±17 (13-56)	17±8 (7-33)	37±11 (10-50)

[a] AI = modified AI system; SI = specially designed instrument.

[b] Based on pregnant recipients.

[c] Two pregnant recipients were slaughtered between Days 35 and 45; it is assumed that these recipients would have farrowed.

[d] Recipients were slaughtered on Day 35 of pregnancy but were assumed to have farrowed.

[1)] Chapter 3

[2)] Chapter 5

Two research groups used systems which need complete anesthesia (Reichenbach et al. 1993, Li et al. 1996). Reichenbach et al. (1993) brought the recipients into a vertical position with their hindquarters raised to 90^0 from the horizontal plane, in order to straighten

the cervical canal and to stimulate the transport of the embryos through the uterus by gravity. These workers used an AI-spirette in combination with a stainless steel embryo transfer canula (as used for ET in cattle), which is inserted through the spirette into the uterine body. The recipients in the study of Li et al. (1996) were positioned on their back, to straighten the cervical canal. The workers also used a modified AI-spirette in combination with a specially designed stainless steel instrument with a testing bar for passing the cervical canal and with additional tubing to deliver the embryos into the uterus. Both groups delivered the embryos into the uterus in a small volume of transfer medium. The first group (Reichenbach et al. 1993) infused an additional volume of 10 to 20 mL of medium into the uterus after deposition of the embryos (Table 1).

Two other research groups used a more traditional modified AI system (Galvin et al. 1994, Yonemura et al. 1996). The recipients were either mildly sedated (Galvin et al. 1994) or not sedated (Yonemura et al. 1996). Both research groups used a normal AI-spirette connected to a three-way stop-cock. Galvin et al. (1994) placed the embryos with a tomcat catheter into the spirette and flushed them with 10 to 12 mL of medium followed by an additional 15 mL of air to empty the spirette into the uterus. Yonemura et al. (1996) flushed the embryos directly with a few charges of fluid (a total of up to 50 mL) into the uterus, followed by 10 mL of air (Table 1).

In our laboratory (Chapter 3 to 6), we used an approach without sedating the recipient sows. The embryos were transferred with a specially designed flexible instrument, which is moved through the cervical canal into the uterus. A small catheter containing the embryos is moved through this instrument into the uterus where the embryos were deposited with a small volume ($\leq$ 0.1 mL) of transfer medium. This procedure resulted in an initial pregnancy rate of 33% (Chapter 3). More recently, using a similar but improved transfer procedure and the transfer of more embryos, a relatively high pregnancy rate of 59% was reported (Table 1; Chapter 5).

The obtained average pregnancy rates have ranged from 9 to 64%, with average litter sizes ranging from 3.1 to 10.9 (Table 1). Although all groups used different approaches for their transfers, the best pregnancy rates were achieved without sedation of recipients (approximately 60%; Yonemura et al. 1996; Chapter 5). Specially designed instruments which enable deposition of embryos into the uterus with a small volume of fluid resulted in higher litter sizes (approximately 6 to 11 piglets; Li et al. 1996, Chapter 3 and 5; Table 1) than the other methods (3 to 5 piglets). Perhaps the low litter size when large volumes of fluid (often combined with additional air to flush the catheter) are used to transfer the embryos is due to

the back-flow of fluid together with the embryos through the cervical canal. In our opinion it is important to use only a minimal amount of fluid to flush the embryos from the catheter; in the uterus very little fluid is normally present during early pregnancy.

FEASIBILITY OF THE TRANSFER PROCEDURES

The feasibility of transfer procedures depends on the ease with which transfers can be performed, using minimal equipment and time, preferably under farm conditions comparable to those for artificial insemination. Thus, transcervical procedures without sedation of the recipients are preferable to endoscopic procedures, which still require surgical facilities. The feasibility of non-surgical procedures also depends on the experience needed to perform such transfers. The introduction of special instruments through the cervical canal of non-sedated recipients is currently used only in weaned sows, because the cervix in gilts is to narrow and more tightly closed (Chapter 3 and 5). However, for all procedures mentioned, trained personnel will be needed to perform the transfers.

FACTORS AFFECTING THE SUCCESS RATE

Most studies were performed at variable stages of embryonic development (4-cell to hatched blastocysts) and with synchronous or 1-d asynchronous recipients. Most authors found no clear difference in results which could be attributed to those factors. Recent research from our laboratory (Chapter 4) indicated that approximately synchronous transfers (recipients ovulating approximately 24 h before to 12 h after the donors) gave the best results, which differ from previously published results for surgical transfers (Polge 1982). In this previous work the results were optimal if the embryos (Day 3 to Day 9) were transferred to recipients which came in estrus 1 or 2 d after the donors. An explanation might be that after surgical transfers the embryos might benefit from such asynchrony due to the use of anesthesia or the infliction of surgical trauma.

The developmental stage of the embryos seems to be important in successful embryo transfer. Better results were found when Day 4 transfers were performed with litters containing blastocysts together with morulae (6/11, 55% pregnant) than with transfers using only morula stage embryos (also Day 4 embryos; 1/10, 10% pregnant; P=0.06; Chapter 3). These results might indicate that only blastocysts will result in good transfer results or that only the best developed litters will survive. Research with Day 5 transfers (5 d after

ovulation) resulted in no pregnancy at all when 5 or more morulae or only morulae were within a transferred litter of morulae and blastocysts, independent of the asynchrony of the recipients (recipients ovulating 24 h before to 36 h after the donors (Chapter 4). These results indicate that litters with relatively advanced embryos have a higher chance of surviving non-surgical transfer to approximately synchronous recipients. Recent research from our laboratory emphasized this phenomenon (Chapter 5). A low and a high dose (1000 vs 1500 iu) of eCG to stimulate the number of ovulations and embryos in donors was compared. The resulting Day 5 embryos (expanding blastocysts) were scored for development and transferred to synchronous recipients. The low and high dose of eCG resulted in differences in cell number per embryo (84±15 vs 70±2; $P<0.05$) and a small difference in average blastocyst diameter (162±12 μm vs 158±11 μm; $P>0.05$). The pregnancy rates were 71 and 46% ($P=0.18$), respectively. On average, categorizing the transferred litters on the basis of their average diameter resulted in a pregnancy rate of 43% for litters with the smaller blastocysts and a pregnancy rate of 77% ($P=0.07$) for litters with the larger blastocysts, emphasizing the importance of a good development of the embryos. Several experiments have substantiated the importance of well-developed embryos for maximal embryo survival using asynchronous transfers. Besides experiments with asynchronous transfers (e.g. Pope et al. 1982), one experiment has been described, reporting the relation between embryonic development and survival within animals under physiological conditions (Wilmut et al. 1986). Embryos were divided in different development groups and transferred to isolated uterine parts, which resulted in increased survival for better developed embryos.

In general it seems that on Day 5 after ovulation only well-developed blastocysts can be transferred successfully by our non-surgical procedure, and that the recipients have to ovulate between 24 h before and 12 h after the donors.

Other aspects that affected the results of non-surgical transfers were reported by Yonemura et al. (1996). They found better results when minimal resistance was observed when their instrument was introduced into the cervix and the PBS-solution was being infused into the uterus. Their technique is based on insertion of the AI catheter as deeply as possible into the cervical canal and then flushing the embryos through the last part of the cervical canal or, by introduction of the catheter deep enough to enter the uterine lumen. The deepest insertion of the instrument, probably into the uterus, resulted in the best results, by preventing the embryos from adhering to the cervical mucus. With the method used in our laboratory no clear difference in pregnancy rates was observed with regard to the duration of the transfer or resistance to the introduction of the instrument (Chapter 6). This might indicate that cervical deposition of the embryos is very unlikely to occur when our procedure is used.

Differences in characteristics of the recipients like age or previous litter size were not related to differences in pregnancy rates (Chapter 6). This indicates that differences in uterine quality, caused by age or related to the previous litter size, do not affect embryo survival after synchronous transfers or that the differences in uterine quality, which might exist at weaning, disappeared during the period (approximately 4 weeks) before embryo transfer. Differences in uterine development between first oestrus and multioestrus gilts have been reported (Murray and Griffo 1976, Schnurrbusch and Erices 1979). Although, embryonic survival after transfer differed between first- and second-oestrus donors (63.8 and 84.9%, respectively; $P<0.05$), no differences in transfer results are found for surgical transfers to first- and third-oestrus recipients (pregnancy rate 67.5 and 60.0%, embryonic survival 76.1 and 78.2%, respectively, $P>0.05$; Archibong et al. 1992). This indicates that the condition of the uterus is less critical than embryonic quality for transfer results.

Another aspect which might possibly affect transfer success might be related to hygiene. The groups with the most successful procedures (Yonemura et al. 1996 and our group: Chapter 5) have used antibiotics in their transfer medium and covered their instruments with sheets to prevent contamination of the uterus with vaginal material. In our last experiments (Chapter 5 and 6), the recipients were also preventively treated with antibiotics from the day of ovulation (5 d before transfer) up to 5 d after the transfer. Whether this is of major importance remains unclear, but at least it does not seem to be harmful for embryonic survival.

Since the requirements of synchrony and embryonic development for successful non-surgical transfers differ from those for surgical transfers, they must be attributed to the differences in the transfer procedure. The 3 main differences between surgical transfers and our non-surgical approach are that in our non-surgical procedure the embryos are collected following slaughter of the donors, the recipients are not sedated and the embryos are transferred into or near the uterine body.

The effect of slaughtering the donors on embryo quality has been previously studied. Embryos collected immediately after slaughter have a higher in vitro development rate than embryos collected 2 h after slaughter (Wollenberg et al. 1990). Results of surgical transfers of such embryos, collected immediately after slaughter, were similar to results of transfers of surgically collected embryos (Schliepper and Holtz 1986). It is, therefore, unlikely that the (quick) collection of embryos after slaughter explains the differences between transfer results from the surgical and non-surgical procedures.

The effect of sedation of the recipients cannot be investigated in surgical transfers, while no comparison has been made in non-surgical transfers. In some species like cattle no sedation is needed, so sedation has probably no positive effect on transfers. Lack of sedation may in fact be beneficial, since the recipients are not stressed by the awakening from anesthesia and negative effects of anesthesia on physiological processes are also prevented. Optimally; non-surgical transfers should be performed without sedation, as long as the procedure is painless to the animals.

The effect of the location of the transferred embryos has also been studied (Stein-Stefani and Holtz 1987, Wallenhorst and Holtz 1995), and no difference in embryonic survival was found between embryos that were transferred to the middle compared with the tip of the uterine horn (Stein-Stefani and Holtz 1987). Survival after surgical transfer of blastocysts to the uterine body resulted in only 2/17 (12%) pregnancies with 4 and 5 fetuses, while transfers to the caudal quarter or tip of the horn resulted in 81% (13/16) and 88% (14/16) pregnancy rates with an average of 5.6 and 8.2 fetuses, respectively (Wallenhorst and Holtz 1995). The poor results of these surgical transfers to the uterine body, which are similar to results reported by Yonemura et al. (1996), are even worse than published results for non-surgical procedures (Table 1). This might indicate that the poor results of surgical transfers to the uterine body (Wallenhorst and Holtz 1995) are negatively affected by more important aspects (e.g., anesthesia or surgical trauma) than just the location of the embryos. However, for non-surgical transfers, it can not be excluded that the location of the embryos in the uterine body or beginning of the uterine horn is suboptimal.

It seems that causes for the differences in the requirements for surgical and non-surgical procedures are attributable to the location of the embryos after transfer, since sedation or the method of embryo collection presumably do not affect embryonic quality.

OBSTACLES

It is clear that several parameters which may affect the success rate of the transfers have not yet been investigated fully. Aspects affecting the transfer results such as location of the embryos in the uterus, embryo developmental parameters of age and development, asynchrony and embryonic collection methods need to be substantiated. Other aspects such as age of donors and recipients (prepubertal or pubertal gilts, sows), synchronization methods for donors as well as recipients and embryo handling methods (storage or culture, medium composition, time and temperature) will all need to be investigated in the future. A negative effect of culturing embryos for a relatively long period of 3 d on subsequent results of embryo transfer has been reported

(Blum-Reckow and Holtz 1991). Transfer to asynchronous (less developed) recipients was needed to improve pregnancy rates from 9 to 53%. Control transfers of fresh embryos resulted in pregnancy rates of 60 and 70% for synchronous and asynchronous transfers, respectively. Synchronous surgical transfers with large geographical distances between donors and recipients, and resulting in a storage period of 30 to 34 h have also been published (Niemann et al. 1989), reporting a pregnancy rate of 47% and a litter size of 5.6 piglets. These results indicate that it is possible to perform successful transfers after rather extended storage periods, although the results might be improved if storage conditions are optimized.

Presently, results of non-surgical transfer procedures seem to be acceptable to use this technique for high value applications. Therefore the procedures have to be tested and optimized in practice. The expertise which will be gained in this way will add information on aspects important to the success of non-surgical embryo transfer.

Another important aspect is the development of sanitary protocols to prevent the introduction or transmission of infectious agents, although hardly any evidence exists that disease transmission has occurred by embryo transfer (Wratthall and Sutmöller 1998). However reliable safety protocols are important to diminish every risk on disease transmission. The experience with cattle and human embryo transfers form a solid basis for development of similar safety protocols for pig embryo transfer. If the right protocols are used, the non-surgical transfer procedures will be safer and preferable above alternative procedures to keep farms closed for animals from other farms. These alternatives are the production and transport of SPF-piglets produced by surgical procedures with its specific disadvantages, or breeding the animals with semen from boars of the desired genetic background, which introduces only 50% of the desired genetic lines.

EMBRYO COLLECTION TECHNIQUES

For embryo transfer in pigs a relatively high number of embryos is needed. The conventional way to obtain embryos is by surgical collection. This has the usual disadvantages of surgical procedures and can be repeated only up to 2 or 3 times due to the formation of scar tissue (Polge 1977, Cameron et al. 1989). However, for specific applications the surgical method can be useful and it has the advantage of high recovery rates. Another method with a high recovery rate is flushing of the uterus after slaughter of the donor. The disadvantage is that donors can be used only once. This factor limits application to donors which are destined for culling, like older sows and prepubertal breeding gilts, which may carry desirable genes which are valuable for breeding. A hygienic collection of embryos at the slaughterhouse can be

guaranteed by special precautions to prevent contact of the animals to be slaughtered with other groups of animals. Additionally, it is possible to collect the intact uterus in a clean aseptically way, and to collect embryos in a separate clean room. Therefore, both collection at the slaughterhouse as well as surgical collection can provide a supply of embryos, especially for small-scale applications.

Other, recently developed techniques are the transcervical collection of embryos from donors with surgically shunted uterine horns (Chapter 1 and 2, Kobayashi et al. 1989) and endoscopic procedures (Besenfelder et al. 1997, Brüssow and Ràtky 1996). The endoscopic technique has the advantage that it is minimally invasive, although it remains a surgical procedure with the accompanying disadvantages. In this way embryos can be collected from the oviduct as well as from the uterus with high recovery rates (28 oocytes or embryos per donor; Besenfelder et al. 1997).

Collection of embryos from donors with shunted uterine horns is based on the surgical connection of the tip of the uterine horns to the base of the horns, near the bifurcation of the uterine body. The large middle portion of the uterine horns is closed and is left in the abdomen (Chapter 1 and 2) or removed (Kobayashi et al. 1989). Transcervical collection procedure from 'permanent' donors with surgically shunted uterine horns has the advantage that every 3 wks embryos can be collected. However, recovery per transcervical collection is lower (6.3±3.4 embryos) than the recovery after slaughter (17.7±2.2; $P<0.05$; Chapter 2). These results are comparable with other published results (6.3±6.0 embryos; Kobayashi et al. 1989). In combination with superovulation induction, the recovery per collection can increase from approximately 8 to 18 embryos (Chapter 2). This technique has had only limited use (merely experimental), and as far as we know no transfer has been performed using transcervically collected embryos.

PROSPECTS FOR PRACTICAL APPLICATION OF EMBRYO TRANSFER

One of the important benefits of embryo transfer is the possibility to transport embryos instead of live animals. Transport of embryos minimizes the risk of disease transmission and diminishes transportation costs and animal stress considerably. Several groups have reported international transport of porcine embryos with subsequent successful embryo transfer, indicating the feasibility of embryo transfer in this context (Baker and Dziuk 1970, Wratthall et al. 1970, Niemann et al. 1989). This offers the possibility for internationally operating pig breeding companies to transport their genetic material all over the world in a safe and cost-effective way.

Both collection and transfer of embryos can be done surgically. The possibility to collect or to produce embryos by less invasive methods and to transfer them to recipients non-surgically will enhance the simplicity, effectiveness and profitability of embryo transfer. The non-surgical procedures will be preferable to surgical procedures from both an animal welfare point of view and because these procedures can be performed on farms without the need for special facilities. The expected improved health status of pig populations and the diminished animal stress when embryos instead of animals are transported, are important for acceptance of these procedures by society. On the other hand, the use of artificial reproductive technologies in animal production is a matter of concern for the public. Therefore, when these artificial reproduction technologies are considered for implementation in practice, the benefits need to be balanced against the possible negative perception by society. The availability of non-invasive embryo transfer procedures might increase the acceptability of these applications.

The lack of a reliable source for large numbers of embryos needs further attention. The procedures of in vivo embryo collection by surgery, or collection from sows with surgically shunted uterine horns will both not be readily accepted by society and are anyway not really convenient, especially to obtain large numbers of embryos for large scale applications. The collection of embryos at the slaughterhouse will probably be more practical and will probably be more readily accepted by society. However, for collecting embryos from sows with a high breeding value slaughtering of these sows will be an expensive procedure. Therefore, substantial efforts are needed to develop methods to produce large numbers of transferable embryos, without using invasive techniques. The most attractive possibility in this respect is to produce embryos by in vitro production (IVP), either by nuclear transfer (cloning) or by in vitro maturation and fertilization of oocytes (IVM-IVF) and subsequent culture of these embryos to the desired stage of development. Both techniques have great potential and are continuously improved (Prather and Day 1998, Stice et al. 1998). It will take some time before they are available for general use. Development and application of related reproductive techniques like (endoscopic) ovum pick-up, gamete and embryo freezing, sexing of sperm cells or embryos, and selection of embryos based on DNA markers can give a great impetus to the application of embryo transfer. An important concern is the development of the piglets born by using these techniques. It is known that after in vitro production of embryos, the development of the resulting offspring can be disturbed, resulting in higher birth weights and, in some cases, anatomical disorders as shown in cattle and sheep (Kruip and Den Daas 1997). If these problems can be prevented, new applications for pig breeders are realizable to increase genetic progress and to ensure the international trade of valuable genetic material (Van der Lende and Hazeleger 1998).

An example of the possible practical application of non-surgical embryo transfer in the future, is the production of grower-finisher pigs. Besides cloning, a more conventional approach might be to inseminate finisher gilts with the semen of finisher boars of another breed to provide crossbred embryos for grower-finisher purposes. An alternative is to produce these embryos by IVP, using oocytes retrieved from such gilts. Theoretically, the number of available embryos is very large, and is limited only by the number of gilts slaughtered. These embryos can be transferred to recipients selected for good mothering abilities. This way of producing grower-finisher piglets may have a large potential on very large farms with own on-farm slaughtering facilities.

At present, the availability of embryos is limited and only small-scale applications of non-surgical embryo transfer are possible. However, the value of these small-scale applications can be considerable for genetically valuable pig populations.

Realization of these applications in practical pig breeding will have consequences for the breeding organizations. The selection of animals, synchronization, timing of inseminations, collection and transfer of embryos have to be performed by trained people, according to strict protocols. This will require much attention and labor, but is unavoidable for a successful implementation of these techniques.

CONCLUDING REMARKS

The results of this study show that it is possible to successfully collect and transfer embryos by transcervical procedures.

To collect embryos in vivo by a transcervical procedure, sows with resectioned uterine horns have to be used. Although the results are rather variable, the procedure might be useful as a research tool or for small-scale applications in practice. Further research on the causes of the large variability in the flushing results and further development of this procedure might make it more efficient. The major disadvantage of this procedure is that the uterus of the sow is modified permanently by surgical procedures. This will make this technique difficult to accept for society.

The non-surgical procedure for embryo transfer which has been developed, has led to results which are acceptable for research and for practical applications. Compared with other transfer procedures it appears to be a relatively feasible procedure, without need for special facilities or (invasive) treatments of recipients. Although, for the moment, the technique is

relatively inefficient (transfer of 30 well developed embryos from approximately 2 donors, resulting in a pregnancy rate of 60% and approximately 10 piglets per recipient) in comparison with a normal pregnancy rate of approximately 90 % and a litter size of 11 piglets, further improvements of the results are expected. From this study it appeared that the embryonic development is important for the success rate of the transfers. Therefore, factors affecting the quality of the embryos and their survival in the uterine body need further attention. Also factors like synchrony between donors and recipients and factors affecting the quality of donors (embryos) and recipients (uterus) need further research. Finding factors that improve the efficiency of non-surgical embryo transfer is important for practical applications, but also gives more insight in factors affecting embryonic survival in general.

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SUMMARY

SUMMARY

Embryo transfer in pigs has always been a valuable tool to study the relation between embryonic development and embryonic survival as well as embryo-uterus interactions. For practical applications it has been used only occasionally. This was due to the specific disadvantages of the existing surgical embryo collection and transfer procedures (i.e. invasive procedures requiring special facilities) and the limited need for practical application of embryo transfer, considering the high fecundity of pigs. However, for export of genetic material (prevention of long distance shipment of animals) and disease control, embryo transfer can be important.

Considering the disadvantages of surgical transfer procedures, transfer of embryos by an uncomplicated non-surgical procedure would be preferable. For a long time non-surgical embryo transfer in pigs has been considered a technique which was almost impossible to perform. The reason was the complex nature of the genital tract with its wrinkled cervical wall and the long and coiled nature of the uterine horns, while the embryos are located in the top of the horns during early pregnancy.

Therefore, the aim of this research was to develop non-surgical procedures for the collection and transfer of porcine embryos which can be used for both practical and research purposes. The development of these procedures was expected to give insight in factors which are important for embryonic survival.

The development of non-surgical procedures for embryo collection and transfer was preceded by the development of a non-surgical procedure, to obtain access to the lumen of the uterine body. The first obstacle thereafter, was how to collect embryos from the uterus of a sow. During early pregnancy the embryos are located in the top of both uterine horns. Each horn is 1 to 2 m long and is positioned in the abdominal cavity in a coiled and twisted way. A solution for this problem is described in Chapter 1. In this chapter a surgical procedure is presented which can be used to permanently shorten the uterine horns by detachment of a large central part of each uterine horn. In this way, the remaining (i.e. shortened) uterine horns can be flushed for embryo collection. The results showed that it was possible to collect embryos from these sows. In Chapter 2 an improved flushing procedure is described and the results of the embryo collection procedures are presented and evaluated. It was possible to collect embryos routinely and their development appeared to be normal. After superovulation induction the (highly variable) yield increased from approximately 8 (range 4 to 19) to 18 (range 0 to 71) embryos.

Subsequent research focussed on the development of a procedure for transcervical transfer of embryos. These embryos were flushed from uteri of sows which were slaughtered. Using this procedure, embryos can be obtained relatively easily and high yields are possible. In this way emphasis could be placed on the development of the non-surgical transfer procedure.

The results of an experiment in which Day 4 embryos were transferred are presented in Chapter 3. On average 17 embryos (morula and early blastocyst stages) per recipient were transferred to 21 recipients. This resulted in 7 pregnancies with on average 6.7 piglets born. Ten of these transfers with the best developed Day 4 embryos (containing early blastocysts among the embryos) resulted in six pregnancies. The conclusion was that the results depended on the developmental stage of the embryos and the possibility that a slight asynchrony between donors (embryos) and recipients affected the results.

Subsequent research was therefore focussed on Day 5 embryos, which might possibly be more competent to survive the uterine environment in or near the uterine body. In this experiment the possible effects of asynchrony were investigated (Chapter 4). Day 5 embryos were collected from spontaneous ovulating donor sows and transferred to 31 spontaneous ovulating recipients which ovulated between 24 h before and 36 h after the donors (checked by ultrasound). The best results were obtained when the embryos were transferred to recipients which ovulated between 24 h before and 12 h after the donors. The embryos transferred to recipients ovulating 18 to 36 h after the donors resulted in no pregnancies at all. The transfer of less developed Day 5 embryos (morula stages) also resulted in no pregnancies at all, even if they were transferred to recipients ovulating 18 to 36 h after the donors. The conclusion was that in non-surgical embryo transfers, the embryos have to be in a relatively advanced developmental stage (blastocyst) and have to be transferred to approximately synchronous recipients.

In a pilot experiment it appeared that of Day 5 embryos (120 h after ovulation) expanded blastocysts had the best survival chances and in the next study only these embryos were used. The next aim was to find descriptive parameters which could indicate the quality and selection criteria for the embryos at the moment of transfer.

In Chapter 5 a low (1000 iu) and high (1500 iu) dose of eCG were used to stimulate the ovulation rate in the donors. The expectation was that the dosage of eCG would affect the number and quality of the embryos and consequently effects on embryonic development and subsequent survival after transfer could be studied. It appeared that the higher dosage increased the number of transferable embryos (expanded blastocysts), but their development

(number of cells per embryo) was reduced. The pregnancy rate after transfer of 30 expanded blastocysts, 71 and 46 % for low and high dosage, respectively, was not significantly different nor the number (on average 11) of foetuses on Day 35 of pregnancy. Independent of the eCG dose, it appeared that transfer of the litters with the on average largest embryos resulted in a pregnancy rate of 77%, while transfer of the on average smaller embryos resulted in a pregnancy rate of only 43% (P=0.07), with an average of 12 and 9 foetuses, respectively (P=0.06). It was concluded that a straightforward parameter like diameter of the embryo, can give valuable information about the survival chances after non-surgical transfer of the embryos.

In Chapter 6 data of a pilot experiment and the data from Chapter 5 were combined to study other factors which might contribute to the success of the transfer procedure. In both experiments only transfers with 30 expanded blastocysts, resulting in similar transfer results (pregnancy rate approximately 60%) were analysed. Characteristics of the donor sows (parity, weight, previous litter size, etc.) were related with the yield of transferable embryos. It appeared that the use of young or old donor sows resulted in a relatively low percentage and number of transferable embryos. Furthermore, donor sows with a large litter size preceding transfer, had the best results in terms of percentage and number of transferable embryos. The characteristics of recipient sows were related with the transfer results, but no relation between these parameters were found. Also characteristics of the procedure of embryo transfer (like the resistance felt at introduction of the instrument in the genital tract, duration of the transfer procedure, etc.), which could contribute to the transfer results were studied. No relation was found between the transfer results and the characteristics of the procedure of embryo transfer.

In the General Discussion, the procedures and results of our studies are summarised and compared to the procedures and results from other groups. Also prospects for practical applications for the pig breeding industry are discussed.

The most important result of the experiments described in this thesis, was that it was possible to collect and transfer embryos by trans-cervical procedures.

For trans-cervical embryo collection, donor sows with resectioned uterine horns are needed. Although the embryo yield is rather variable, the procedure might be useful as a research tool or for small-scale applications in practice. The major disadvantage of this procedure is that the uterus of the sow is modified permanently by surgical procedures. Therefore some other possibilities for embryo collection are discussed.

The developed non-surgical transfer procedure appeared to be feasible and the results are acceptable for research and some practical applications. The results (approximately 60% pregnant and 11 foetuses on Day 35 of pregnancy) are comparable to the results of the

classical surgical procedures. In comparison to most other non-surgical procedures our procedure has the advantage that no sedation of the animals is needed. For the moment, the technique is still relatively inefficient; 30 well developed embryos are transferred, resulting in a pregnancy rate of 60%. It appeared that the embryonic development at the time of transfer is important for the success rate of the transfers. Therefore, factors affecting the quality of the embryos and their survival after transfer in the uterine body need further attention. Finding factors that improve the efficiency of non-surgical embryo transfer is important for practical applications, but also gives more insight in factors affecting embryonic survival in general.

SAMENVATTING

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Embryo-transplantatie is altijd een waardevolle techniek geweest om de relatie tussen embryonale ontwikkeling en overleving en embryo-uterus interacties te bestuderen. Voor praktische toepassingen bij varkens is het slechts af en toe gebruikt. Dit is te wijten aan de specifieke nadelen van de bestaande chirurgische procedures om embryo's te verzamelen en te transplanteren (d.w.z. invasieve procedures, waarvoor speciale faciliteiten nodig zijn) en de beperkte noodzaak voor praktische toepassing van embryo-transplantatie vanwege de grote vruchtbaarheid van varkens. Echter, voor export van genetisch materiaal (het voorkomen van langdurige transporten van dieren) en ziektebeheersing kan embryo-transplantatie belangrijk zijn.

Gezien de nadelen van de chirurgische procedures, zou een eenvoudige niet-chirurgische procedure voor het transplanteren van varkens-embryo's wenselijk zijn. Gedurende lange tijd is niet-chirurgische embryo-transplantatie beschouwd als een techniek die bijna onuitvoerbaar was. De redenen hiervoor waren de ingewikkelde structuur van het geslachtsapparaat van zeugen met zijn geplooide cervixwand en de lange en gekrulde vorm van de uterushoornen, terwijl de embryo's zich tijdens de vroege dracht in de top van de hoornen bevinden.

Het doel van dit onderzoek was daarom om niet-chirurgische procedures te ontwikkelen voor het verzamelen en transplanteren van varkensembryo's, die gebruikt kunnen worden voor zowel praktijk- als onderzoeksdoeleinden. Verwacht werd dat de ontwikkeling van deze procedures inzicht zouden geven in factoren die van belang zijn voor de embryonale overleving.

De ontwikkeling van niet-chirurgische procedures voor het verzamelen en transplanteren van embryo's werd voorafgegaan door de ontwikkeling van een procedure en instrument om via de cervix in het uterus-lumen te kunnen komen. De eerste moeilijkheid daarna was hoe de embryo's uit de uterus van een zeug gespoeld konden worden. Tijdens de vroege dracht bevinden de embryo's zich in de top van beide uterushoornen. Elke hoorn is 1 tot 2 meter lang en bevindt zich gekruld en gedraaid in de buikholte. Een oplossing voor dit probleem is in Hoofdstuk 1 beschreven; met behulp van een chirurgische procedure worden uterushoornen permanent te verkort door een groot middendeel van elke hoorn weg te halen. Op deze manier kunnen de verkorte hoornen gespoeld worden om de embryo's te verzamelen. De resultaten toonden aan dat het mogelijk was om embryo's van deze zeugen te verzamelen. In Hoofdstuk 2 is een verbeterde spoelprocedure beschreven en zijn de resultaten van het

embryo's verzamelen gepresenteerd en geëvalueerd. Het bleek mogelijk embryo's routinematig te verzamelen en hun ontwikkeling bleek normaal te zijn. Na superovulatie-inductie nam de (erg variabele) opbrengst toe van ongeveer 8 (range 4 tot 19) tot 18 (range 0 tot 71) embryo's, hetgeen betekent dat gemiddeld ongeveer de helft van de aanwezige embryo's op deze wijze te winnen zijn

Vervolgonderzoek richtte zich op de ontwikkeling van een procedure voor trans-cervicale transplantatie van embryo's. Deze embryo's werden uit de uterus van geslachte zeugen gespoeld. Op deze wijze kunnen embryo's op een relatief eenvoudige wijze verkregen worden en zijn hoge opbrengsten mogelijk, waardoor de aandacht gericht kon worden op de ontwikkeling van de niet-chirurgische transplantatieprocedure.

De resultaten van een experiment waarin embryo's met een leeftijd van 4 dagen werden getransplanteerd, zijn gepresenteerd in Hoofdstuk 3. Gemiddeld werden 17 embryo's (morula en blastocyst stadia) per ontvangster getransplanteerd naar 21 ontvangsters. Dit resulteerde in 7 drachten met gemiddeld 6,7 geboren biggen. De tien transplantaties met de best ontwikkelde Dag 4 embryo's (met vroege blastocysten tussen de embryo's) resulteerden in 6 drachten. De conclusie was dat de resultaten afhankelijk waren van het ontwikkelingsstadium van de embryo's en de mogelijkheid dat een beperkte asynchronie tussen donoren (embryo's) en ontvangsters de resultaten beïnvloedde.

Vervolgonderzoek werd daarom gericht op Dag 5 embryo's, die mogelijk beter in staat waren om in het milieu van de plaats van transplantatie (het uteruslichaam en nabije omgeving) te overleven. In dit experiment werden mogelijke effecten van asynchronie onderzocht (Hoofdstuk 4). Dag 5 embryo's werden verzameld van spontaan ovulerende donoren en getransplanteerd naar 31 spontaan ovulerende ontvangsters, die ovuleerden tussen 24 uur voor en 36 uur na de donoren (gecontroleerd m.b.v. echografie). De beste resultaten werden verkregen indien de embryo's getransplanteerd werden naar ontvangsters die tussen 24 uur voor en 12 uur na de donoren ovuleerden (uterusmilieu loopt 24 uur voor tot 12 uur achter op de embryo's). De embryo's die getransplanteerd werden naar een uterusmilieu (ontvangsters) dat 18 tot 36 uur achterloopt op de embryo's resulteerden in geen enkele dracht. De transplantaties van minder ontwikkelde Dag 5 embryo's (morula stadia) resulteerden eveneens in geen enkele dracht, zelfs niet indien ze in een 18 tot 36 uur achterlopend uterus milieu werden getransplanteerd werden. De conclusie was dat bij niet-chirurgische transplantaties de embryo's zich in een relatief voorlopend ontwikkelingsstadium (blastocyst) moeten bevinden en dat ze naar ongeveer synchrone ontvangsters getransplanteerd moeten worden.

In een pilot-experiment bleek dat van de Dag 5 embryo's (120 uur na ovulatie) de 'expanded' blastocysten de beste overlevingskansen hadden en in een vervolgstudie werden daarom alleen deze embryo's gebruikt. Het doel van dit experiment was om parameters te vinden die de kwaliteit en daarmee selectiecriteria voor embryo's op het moment van transplanteren konden beschrijven. In Hoofdstuk 5 werden een lage (1000 iu) en een hoge (1500 iu) dosis eCG gebruikt om de ovulatiegraad van de donoren te stimuleren. De verwachting was dat de dosering eCG het aantal en de kwaliteit van de embryo's zou beïnvloeden, zodat effecten op de embryonale ontwikkeling en vervolgens de overleving na transplantatie bestudeerd konden worden. Het bleek dat de hogere eCG dosering het gemiddelde aantal transplanteerbare embryo's (expanded blastocysten) verhoogde van 13 naar 21, maar hun ontwikkeling (aantal cellen per embryo) was verminderd van 84 naar 70. Na 27 transplantaties met 30 expanded blastocysten was het percentage dracht van 71 en 46 voor respectievelijk de lage en hoge dosering niet significant verschillend. Ook het aantal foetussen op Dag 35 van de dracht (gemiddeld 11) verschilde niet tussen de lage en hoge dosering. Onafhankelijk van de eCG dosering bleek dat transplantatie van tomen met gemiddeld de grootste embryo's resulteerden in 77% dracht, terwijl transplantaties met de gemiddeld kleinere embryo's resulteerden in 43% dracht (P=0.07), met een gemiddelde van respectievelijk 12 en 9 foetussen (P=0.06). Er werd geconcludeerd dat een eenvoudige ontwikkelingsparameter als diameter van het embryo, waardevolle informatie kan geven over de overlevingskansen van de Dag 5 embryo's na niet-chirurgische transplantatie.

In Hoofdstuk 6 werden de gegevens van een pilot-experiment en de gegevens van Hoofdstuk 5 gecombineerd om andere factoren te bestuderen die zouden kunnen bijdragen aan de succeskansen van de procedure. Van beide experimenten werden alleen de transplantaties met 30 expanded blastocysten geanalyseerd. De resultaten van beide experimenten waren vergelijkbaar (ongeveer 60% dracht). Kenmerken van donorzeugen (pariteit, gewicht, vorige worpgrootte, etc.) werden gerelateerd aan de opbrengst aan transplanteerbare embryo's. Het bleek dat het gebruik van jonge (pariteit <5) of oude (pariteit >8) donorzeugen resulteerde in een relatief laag percentage (respectievelijk 63 en 56%) en aantal (respectievelijk 13 en 15) transplanteerbare embryo's in vergelijking met zeugen van pariteit 5 tot 8 met 19 (74%) transplanteerbare embryo's. Verder hadden donorzeugen met een grote worp (14 of meer biggen) voorafgaand aan de transplantatie, de beste resultaten ten aanzien van het percentage (81%) en aantal (21) transplanteerbare embryo's. Ook de kenmerken van ontvangsterzeugen werden gerelateerd aan de transplantatieresultaten, maar er werden geen relaties gevonden. Ook de kenmerken van de uitvoering van de embryo-transplantatie (zoals de weerstand die gevoeld wordt bij het inbrengen van het instrument in

het geslachtsapparaat, duur van de transplantatieprocedure, etc) waren niet gerelateerd aan de transplantatieresultaten.

In de Algemene Discussie worden de procedures en resultaten van de studies samengevat en vergeleken met de procedures en resultaten van andere groepen. Ook worden vooruitzichten voor praktische toepassingen voor de varkenshouderij bediscussieerd.

Het belangrijkste resultaat van de experimenten die in dit proefschrift beschreven zijn, was dat het mogelijk bleek om embryo's door middel van trans-cervicale procedures te verzamelen en te transplanteren.

Voor trans-cervicaal verzamelen van embryo's zijn zeugen met verkorte uterushoornen nodig. Hoewel de opbrengst vrij variabel is, kan de procedure bruikbaar zijn als onderzoeksinstrument of voor kleinschalige toepassingen in de praktijk. Het grootste nadeel van deze procedure is, dat de uterus permanent veranderd is door chirurgische procedures. Daarom zijn enkele andere mogelijkheden om embryo's te verzamelen bediscussieerd.

De ontwikkelde niet-chirurgische transplantatieprocedure bleek uitvoerbaar te zijn en de resultaten zijn acceptabel voor onderzoek en enkele praktische toepassingen. De resultaten (60% dracht en 11 foetussen op Dag 35 van de dracht) zijn vergelijkbaar met de resultaten van de klassieke chirurgische procedures. In vergelijking met de meeste andere niet-chirurgische procedures, heeft onze procedure het voordeel dat geen sedatie van de dieren nodig is. Momenteel is de methode nog relatief inefficiënt; 30 goed ontwikkelde embryo's worden getransplanteerd, resulterend in 60% kans op dracht. Het bleek dat de embryonale ontwikkeling op het tijdstip van transplanteren belangrijk is voor de succeskansen van de transplantaties. Daarom verdienen factoren die de kwaliteit van de embryo's en hun overleving in het uteruslichaam beïnvloeden, extra aandacht. Het vinden van factoren die de efficiëntie van niet-chirurgische embryo-transplantatie verbeteren is belangrijk voor praktische toepassingen, maar geeft ook meer inzicht in factoren die de embryonale overleving in het algemeen beïnvloeden.

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CURRICULUM VITAE

Wouter Hazeleger (geboren op 7 oktober 1950 te Barneveld) groeide op in Kootwijkerbroek op een gemengd boerenbedrijf. Hij bezocht de MULO in Barneveld en behaalde het diploma MULO-B in 1967. Daarna bezocht hij de laboratoriumschool te Apeldoorn en behaalde in 1969 het diploma laborant. In dat jaar startte hij zijn loopbaan bij het Research Centrum voor de Kalkzandsteenindustrie te Barneveld, met chemisch-fysisch onderzoek naar de eigenschappen van kalkzandsteen. Dit duurde, met een korte onderbreking voor zijn militaire dienstplicht, tot 1974. In die periode behaalde hij (middels een PBNA-cursus) zijn diploma leerling-analist (1970) en zijn diploma HBO-A (1973; chemie, specialisatie chemische analyse) middels een avondopleiding bij de School voor Laboratoriumpersoneel te Amersfoort.

In 1974 begon hij zijn werk als laboratoriummedewerker voortplantingsonderzoek bij de vakgroep Veeteelt van de Landbouwhogeschool te Wageningen. In hetzelfde jaar trouwde hij met Joke van Hamersveld. In 1979, 1981 en 1984 werden hun kinderen, respectievelijk Gerard, Corine en Willy geboren. Zijn opleiding vervolgde hij via een avondopleiding bij de STOVA te Wageningen en in 1979 behaalde hij zijn diploma HBO-B (chemie, specialisatie biochemie). Zijn werkzaamheden bij de vakgroep waren in eerste instantie gericht op het verrichten van hormoonbepalingen in urine van hoogdrachtige koeien vanwege de mogelijke relatie met moeilijke- en doodgeboortes van kalveren. Daarna volgde een periode met, onder meer, onderzoek naar bronstsynchronisatie en partusinductie bij schapen. Na de oprichting van de "Werkgroep Vroege Dracht" werden zijn werkzaamheden gericht op verschillende aspecten van de vroege dracht bij het varken. Geleidelijk ontstonden tijdens zijn werkzaamheden ideeën voor het ontwikkelen van een niet-invasieve manier om varkensembryo's te verzamelen en te transplanteren, wat mogelijk van belang zou kunnen zijn voor het vroege dracht onderzoek. Uiteindelijk heeft dit geresulteerd in dit proefschrift. Na het behalen van zijn doctorstitel zal hij zijn loopbaan voortzetten als universitair docent bij de leerstoelgroep Gezondheidsleer en Reproductie van de Landbouwuniversiteit Wageningen.