Design, development, and application of a non-surgical deep uterine embryo transfer technique in pigs


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**Implications**

- There is enormous potential for the use of embryo transfer in pig production because it should make it possible to move genetic material (i.e., embryos) with minimal risk of disease transmission, reduced transportation costs, and no effect on animal welfare during transport.
- Unlike other livestock, the commercial use of embryo transfer in pigs has been very limited because embryo collection and transfer require surgical procedures.
- Recently, a new and unique procedure for the non-surgical transfer of porcine embryos deep into a uterine horn of non-sedated gilts and sows has been developed.
- The excellent reproductive performance of recipients following non-surgical transfer of fresh embryos and the promising results obtained with vitrified embryos represent a fundamental advance in the widespread commercial use of embryo transfer by the pig industry.

**Key words:** deep-uterine catheterization, embryos, non-surgical embryo transfer, pig, vitrification

**Introduction**

Embryo transfer (ET) is a procedure that involves the recovery of embryos from the reproductive tract of genetically superior donor females and the transfer of these embryos into the uterus of recipient females for the birth of live offspring. This technology has numerous important applications in farm animal production, including genetic improvement and the national and international movement of genetic material (i.e., embryos) with minimal risk of disease transmission, reduced transportation costs, and no effect on animal welfare compared with the transport of live animals. In addition, ET is fundamental for other biotechnologies related to the in vitro production and manipulation of embryos. Despite these important applications, the commercial use of ET widely varies among species. While ET has been extensively used for commercial purposes in cattle for more than 30 years, its use in ovine, caprine, and particularly porcine species is currently very limited or nonexistent in contrast to other biotechnologies (Rodriguez-Martinez, 2007).

One of the most important factors in the ability to exploit ET technology at a commercial level is the ability to collect the embryos from the donors and to transfer them to the recipients using procedures that can be easily performed under field conditions. As stated by Betteridge (2006), “the simpler the technique, the wider its applicability.” A clear example of this statement is the evolution of ET in cattle, which has been excellently reviewed by Betteridge (2003). In the 1950s, the first efforts to transfer embryos into the uterus of a recipient with a non-surgical procedure were ineffective (Lamming and Rowson, 1952). At that time, surgical methods for embryo collection and transfer in cows were well developed. However, ET did not grow in popularity until non-surgical methods were developed. By the 1960s, the first calf born using non-surgical ET was obtained (Mutter et al., 1964), and new methods of depositing embryos using non-surgical procedures were reported (Rowson and Moor, 1966). During the 1970s, non-surgical methods for collection and transfer completely replaced surgical methods, and the procedures were improved with the development of different non-surgical catheters and protocols. These investigations resulted in a non-surgical, practical procedure for ET in cattle, leading to an explosive increase of commercial ET, today a booming worldwide business.

Currently, ET of in vivo (or in vitro) produced embryos to the uterus of a recipient cow is easy and reliably performed by transcervical intrauterine deposition, with > 60% of pregnancy rates (reviewed in Rodriguez-Martinez, 2012). A major factor contributing to this development was the practical necessity of avoiding surgery if ET was to become practicable in the field. Necessity really was the mother of invention (Betteridge, 2003). At least 100,000 donor females were flushed in 2010, with more than 730,000 embryos collected and almost 600,000 embryos transferred, 55% of which were frozen. In contrast, the number of sheep and goat embryos collected in that year was less than 5% and 0.1%, respectively, of that recorded for cattle. Unfortunately, no swine ET activity was reported by any country in 2010, although the numbers of flushings reported for 2008 and 2009 in the US were 134 and 9, respectively. In addition to commercial and market factors, current ET in small ruminant and pigs are performed...
using surgical (laparotomy and/or laparoscopy) procedures due to the special anatomy of the female genital tract, which precludes its practical use under field conditions. The development of practical non-surgical procedures to collect and transfer the embryos could allow the commercial use of ET in these species.

This review will describe the difficulties that we encountered during the development of a non-surgical deep uterine (NsDU) ET procedure in pigs. In addition, we will provide results from our laboratory when fresh and cryopreserved embryos are transferred using this procedure.

Why Was it Important to Develop a Procedure for NsDU-ET in Pigs?

In the 1960s, laparotomy appeared as the best method to collect and transfer pig embryos, although Polge and Day (1968) demonstrated that pregnancy could be established by non-surgical ET. Despite this finding, non-surgical ET was considered an impossible technique for many years because of the complex anatomy of the swine genital tract (Figure 1). However, in the 1990s, several non-surgical techniques to deposit embryos directly into the uterine body were developed, although most of them were not successful. Farrowing rates ranging between 5 and 41% and litter sizes ranging between 5 and 7.5 piglets were published (reviewed by Hazeleger and Kemp, 2001; Martinez et al., 2005). The most promising technique for commercial non-surgical ET was developed by Hazeleger and colleagues, who devised a very interesting procedure to deposit the embryos directly into the uterine body of non-sedated sows using a specially designed flexible instrument (Hazeleger and Kemp, 1994). Using this device under field conditions, a farrowing rate of 40% was obtained with > 7 piglets born (Ducro-Steverink et al., 2004). Although these results were close to those obtained surgically (James et al., 1980; Cameron et al., 1989; Niemann et al., 1989), further improvements were necessary to increase the reproductive performance of the recipients after non-surgical ET. The main limitation of the Hazeleger procedure was that they only could reach the uterine body with their catheter, and the embryos had to be deposited in this segment of the uterus, far from the physiological area where embryos enter the cavity (the portions closest to the ovaries, and from where embryos are usually also collected for ET, from the tip of the uterine horns at the morula and blastocyst stages, respectively; Figure 2). In agreement with this hypothesis, the results from surgical ET have indicated that the uterine body is a less appropriate place for deposition of the transferred embryo than the middle or anterior quarter of the uterine horn (Wallenhorst and Holtz, 1999), and thus ET ought to be more adequate if done in these locations. To overcome this and other limitations, in 1998, we designed a research program directed towards developing a new procedure for the non-surgical transfer of porcine morulae and blastocysts deep into a uterine horn of non-sedated gilts and sows.

How Did the Idea of Deep Uterine Catheterization Arise?

The first obstacle to non-surgical trans-cervical introduction of a catheter into the depth of a uterine horn is the cervix (Figure 1), which changes consistency during the estrous cycle (Kunavongkrit et al., 1983). During estrus, the vaginal segment of the cervix and the corresponding cervical folds are constricted and rigid, respectively. The situation is reversed during metestrus, where the segment is relaxed and the folds are smooth (Smith and Nalbandov, 1958). These circumstances make the insertion of a catheter through the vaginal portion of the cervix during metestrus easy to perform. However, before reaching the uterus, the catheter must pass through the uterine segment of the cervix, a region of 4 to 5 cm in length, which is always constricted and where the cervical folds are more closely packed than those in the vaginal portion (Rigby, 1967). Fortunately, the uterine region of the cervix is easily distended during metestrus. The length (60 to 200 cm) and coiled nature of the uterine horns is the last barrier to successful insertion of a catheter into the middle or anterior portion of an uterine horn.

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Figure 1. Reproductive tract of a sow during metaestrus (left). Internal wall of the uterine body (A), uterine (B), and vaginal (C) regions of the cervix (right). The cervical folds in the uterine region of the cervix are more closely packed than those in the vaginal portion.

Figure 2. Chronology of porcine early embryonic development in relation to the day of the estrous cycle.
In 1999, our laboratory developed a fiber-optic endoscopic technique for NsDU insemination in sows without sedation of the animal (Martinez et al., 2001a). This procedure could be performed without any particular technical difficulties because the endoscope has the necessary propulsion force to pass through the cervix and the required flexibility to progress along the uterine horn without perforating the uterine wall (Figure 3). The endoscope was inserted through an artificial insemination (AI) spirette, moved through the cervical canal, and propelled forward along one uterine horn until the entire endoscope was inserted. This procedure could be performed safely and quickly, making it possible to reach the depth of one uterine horn in approximately 97% of the sows in less than five minutes. Unfortunately, the endoscope is an expensive and fragile instrument and, therefore, unsuitable for use under field conditions. However, because an optical system is not necessary to insert the instrument through the cervix and into one uterine horn, we designed a new flexible catheter on the basis of the propulsion force and flexibility of the endoscope. The results obtained using the flexible catheter confirmed the previous findings using endoscopic techniques and demonstrated that the flexible catheter could be inserted during the estrus period in 95% of sows in less than four minutes (Martinez et al., 2002).

What is the Safety and Effectiveness of the NsDU-ET Procedure?

We evaluated the progression force and flexibility of many catheter prototypes. Depending on these characteristics, the main problems detected during the insertions of the catheters were the impossibility of passing the cervical canal, the presence of kinks or loop formations in the cervix or the uterine horn, and the incidence of perforations of the cervical or uterine walls. Finally, we made a flexible catheter with an adequate balance between force and flexibility, and we designed a specific tip to avoid perforations. Currently, the NsDU-ET catheter has a length of 1.8 m, an outer diameter of 4 mm, and a working canal of 0.7 mm, and it is commercially handled by Minitube (DeepBlue Porcine ET Catheter).

Efficiency of the insertions

Intrauterine insertions of the catheter can be performed in regular AI-crates at days 4 to 6 of the cycle in non-sedated recipients. One factor to consider in non-surgical ET programs is the type of recipient used. This catheter can pass the cervical canal and reach the depth of a uterine horn in 90 and 95% of either gilts or sows, respectively, without cervical and/or uterine perforations being reported (Martinez et al., 2004). The cervical canal is obviously more difficult to pass in gilts than in sows, indicating a differential cervical distensibility following the first parity. The principal obstacle in gilts is passing the last two cervical folds, which are often tightly closed. In addition, the number of previous estrous cycles of the gilts seems to be a key factor to obtain adequate success of the insertions. It was possible to insert the catheter through the cervix of any gilt at its second estrus, in contrast with the insertion success rates (> 85%) obtained in gilts with more than two previous estruses (Cuello et al., 2005). A sudden change in cervical distensibility may occur between the second and the third estrus, but the reason remains unknown. The situation is very different when the catheter insertions are performed in weaned sows. In most of them, the catheter advances easily through the cervix to the

![Figure 3. Fiber-optic endoscope used for non-surgical deep uterine insemination (NsDU-AI) in pigs. The endoscope has the necessary propulsion force to pass through the cervix and the required flexibility (A) to progress along the uterine horn.](image)

![Figure 4. Catheter developed for non-surgical embryo transfer (NsDU-ET) in the ad-ovarian segment of one uterine horn of gilts and sows at Days 4 to 6 of the estrous cycle. Note that the tip of the ET-catheter is larger than that for the AI-catheter to minimize damage to the cervical or uterine wall (A).](image)
uterus; it is difficult to feel the passage of the catheter through the cervical folds in some sows, mainly in sows with more than three parities due to the widening of the cervix as more litters are born.

**Evaluation of the position of the NsDU-ET catheter**

For the procedure to be successful, it is not only essential that the catheter be introduced through the cervix into the uterus but also that it is correctly positioned in the uterine horn (Figure 5). Several studies have been performed on this subject. One study used radiographs on non-sedated sows placed in a methacrylate cage to take lateral views of the abdominal cavity (Gomis et al., 2012a). The catheter displayed metal opacity and drew a radiographic pattern characterized by a straight line when it was located into the cervix and the uterine body. In the uterine horn, the catheter formed three or four spiral loops, showing a total adaptation to the anatomy (Figure 6). Correct patterns were observed in 93% of the sows regardless of the day of the estrous cycle. Two sows showed an incorrect pattern due to the formation of a kink in the catheter during insertion (Figure 6). These results confirmed our previous studies (Martinez et al., 2004; Cuello et al., 2005), where it was possible to insert the flexible catheter appropriately into one uterine horn in 80 to 90% of gilts (as determined by laparotomy or after slaughter) and 90% of sows (determined by the shape of the catheter after removal, whether it was straight or bent).

**Prediction of successful catheter placement**

Because a number of catheter insertions are incorrect, an important factor in maximizing the efficiency of this procedure is the ability to ascertain the precise location of the catheter during the insertion. In our earlier studies (Martinez et al., 2004; Cuello et al., 2005), more than 95% of the transfers were correctly positioned, regardless of the type of recipient, which indicated a negligible waste of embryos due to incorrect transfers. Therefore, the embryos should only be introduced into the catheter when the operator is absolutely sure that the insertion is appropriate, so the loss of embryos as a consequence of bad insertions is minimal.

**Depth of catheter insertions**

Previous research showed a poor pregnancy rate when embryos were surgically deposited in the uterine body compared with the caudal quarter or middle of the uterine horn (Wallenhorst and Holtz, 1999). These results demonstrated that the uterine body is a suboptimal site for 5- to 6-day-old embryos and suggests that a procedure to deposit the embryos deep into a uterine horn might increase the farrowing rates and litter sizes achieved after non-surgical ET into the uterine body. With the NsDU catheter, it is possible to reach the second and the third quarter of the uterine horn in 90% of the recipients (Cuello et al., 2005), with no differences between gilts and sows (Martinez et al., 2004). Although this location might be appropriate for embryo deposition, at least when fresh embryos are used, more research is needed to determine whether the uterine placement of transferred embryos has an influence on fertility after NsDU-ET.
Other factors

Several important factors that influence the effectiveness and potential of the NsDU procedure must be considered, including the difficulty of the catheter insertion, the welfare of the animals, and the susceptibility of the recipients to infection after transfers.

In a high percentage of gilts (70%) and sows (80%), none or minor difficulties were encountered during the insertion of the catheter. The time required to complete the procedure was less than 3 minutes, with no differences between gilts and sows. The degree of difficulty encountered in inserting the catheter was not associated with either the behavior of the female or the day of the estrous cycle. In addition, the behavior of the females during the procedure was classified as good or moderate in more than 90% of females, although it tended to be worse in gilts compared with sows. Together, these data suggest that the procedure is basically painless and well tolerated by the recipients, even when the difficulty of inserting the catheter is elevated. In addition, the procedure is non-traumatic, with no bleeding during or after catheter insertions, which suggests that the potential injury or damage to the reproductive tract is minimal.

What Results Can be Expected following NsDU-ET of Fresh or Cryopreserved Embryos?

Using fresh embryos

Currently, we are developing an NsDU-ET program in purebred Duroc sows under field conditions through a private company in northeastern Spain. In this program, high farrowing rates (80%) and litter sizes (9.5 piglets) were obtained when 25 to 30 fresh morulae and blastocysts were transferred to recipients (Figure 7). In addition, we have evaluated factors that would affect the success and practical application of the procedure, including the use of embryos from superovulated donors, the synchrony between donors and recipients, and the use of short-term (24 hour) stored fresh embryos. The donor:recipient ratio can be decreased by inducing superovulation of the donors with 1,000 IU eCG. Using this treatment, the number of donors required to transfer 30 embryos per recipient can be decreased from 2 to 1.5 in purebred Duroc and Pietrain and from 1.3 to 0.8 in hyperprolific lines, with no differences between the control and the superovulated embryos in farrowing rates and litter sizes after NsDU-ET. We have also determined that a recipient asynchrony of +24 hours (recipient in estrus 24 hours after donors) is ideal for successful NsDU-ET.

On the other hand, since embryos must be transported from the donor farm to the recipient farm, a storage period is necessary. In some circumstances, a period of 24 hours is enough to enable such transport. One method to maintain the developmental capacity of the embryos for short periods is in vitro culture. Using this method, acceptable farrowing rates (50 to 60%) and litter sizes (5 to 8 piglets born) were obtained...
after surgical transfers of embryos cultured for 30 hours (James et al., 1980; Niemann et al., 1989). In a recent study, we demonstrated that high reproductive performance in the recipients can be obtained using fresh cultured morulae and early blastocysts kept at 37°C for at least 24 hours in a chemically defined medium after NsDU-ET. Two important conclusions were obtained from this study. First, unlike the controls, none of the embryos cultured in the experimental group hatched at the end of the culture, which is essential because embryos must be protected by an intact zona pellucida for sanitary reasons; second, the embryos could be collected, handled, cultured, and transferred in a chemically defined medium. This fact is essential because embryo culture media usually contain serum or serum components, which carries a risk of disease transmission (Guerin et al., 1997), an important limitation for embryo movement. Based on the results described in this and an earlier study (Martinez et al., 2004), current the NsDU-ET procedure offers new possibilities for its commercial use under field conditions.

Using cryopreserved embryos

The cryopreservation of pig embryos has largely been ineffective due to their high sensitivity to chilling injury, another key limitation to the extensive use of ET in pigs. In addition to the application of ET technology, the development of a procedure to cryopreserve pig embryos would provide transcendentinal advantages for the pig industry, including indefinite embryo storage, maternal germplasm storage, genetic line regeneration or proliferation, increased selection pressure in select herds, rescue of premium genetic stock from diseased herds, improvement or elimination of quarantine conditions, and international export/import of potential breeding stock (Dobrinsky, 2001).

Among different cryopreservation strategies, vitrification is considered the only suitable method for long-term storage of untreated porcine morulae and blastocysts. Post-warming in vitro survival rates of 40 to 70% were initially reported using the open pull straw (OPS) vitrification method (reviewed by Berthelot et al., 2003; Cameron et al., 2006). With the advancements and refinements of vitrification protocols that have been achieved in the last five years, our laboratory and others (reviewed by Martinez et al., 2013) have obtained high post-warming in vitro survival of untreated morulae (>80%) and blastocysts (>90%), which confirms previous findings that embryo pre-treatments before vitrification are not necessary (Cuello et al., 2010). Despite these excellent in vitro results, there are few recent studies on the post-warming in vivo survival of vitrified embryos in the literature (reviewed by Martinez et al., 2013). However, several of the extant studies indicate that it is possible to achieve high farrowing rates (75%) and litter sizes (8.2 to 9.9 piglets born) with these embryos after surgical transfer to recipients.

The combined use of NsDU-ET and embryo vitrification should be fundamental to the widespread use of ET by the pig industry. Our laboratory has recently improved both technologies. We have demonstrated that a chemically defined medium can be used for embryo collection and handling, vitrification, warming, and transfers, and this medium does not require CO₂ pre-incubation (Sanchez-Osorio et al., 2010; Cuello et al., 2013). We simplified the conventional three-step warming procedure and developed a direct (one-step dilution) warming-in-syringe procedure for morulae and blastocysts vitrified by OPS (Cuello et al., 2004; Sanchez-Osorio et al., 2008). With this procedure, an acceptable farrowing rate (42.9%) was achieved, with 5.4 piglets born (Cuello et al., 2005). Unfortunately, a warming-in-syringe procedure appeared harmful to embryos vitrified by the Super OPS (SOPS) method, which is otherwise currently used in our laboratory for its excellent in vitro survival rates. Using this warming-in-syringe procedure with embryos vitrified by the SOPS method led to extremely low pregnancy rates after NsDU-ET (one pregnancy out of 25 transfers; unpublished results).

Recently, refinements of the one-step warming method have resulted in promising farrowing rates (50%) and litter sizes (10.4) after NsDU-ET of SOPS-vitrified morulae and blastocysts (Gomis et al., 2012b). However, farrowing rates are still less than those achieved using surgical procedures, even though more than 80% of the warmed embryos are viable when collected 24 hours after NsDU-ET and almost all of them survive after 48 hours of in vitro culture (Gomis et al., 2012b). Additional studies, including the effect of the number of embryos per transfer, type of recipient, and administration of anti-prostaglandins to the recipients, are necessary to improve the farrowing rates after the NsDU transfer of vitrified embryos.

Conclusions

The procedures outlined in this review describe a unique technique for the non-surgical insertion of a transfer catheter deep into a uterine horn of gilts and sows, which was considered impossible for more than 30 years because of the complex anatomy of the cervix and uterine horns in this species. The development of adequate and practical instrumentation has overcome the anatomical obstacles and has made non-surgical embryo transfer in pigs possible. The transfer procedure is simple, safe, quick, and well tolerated by the recipients. The excellent reproductive performance post-transfer of fresh embryos and the promising results obtained with vitrified embryos represent a fundamental advance in the widespread commercial use of ET by the pig industry, which will benefit from the numerous applications of this technology, particularly from the exchange of relevant genetic material with minimal risk of disease transmission.

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