An update on Reproductive Technologies with Potential Short-Term Application in Pig Production

EA Martinez¹, JM Vazquez¹, J Roca¹, C Cuello¹, MA Gil¹, I Parrilla¹ and JL Vazquez²
¹Department of Medicine and Animal Surgery, University of Murcia, Murcia, Spain; ²Department of Surgery, University Miguel Hernández, Elche, Spain

Contents
Over the past decade, there has been an increase in the development and/or in the improvement of emerging reproductive technologies in pigs. Among emerging reproductive technologies with potential short-term application in pig production are: artificial insemination with low number of spermatozoa, cryopreservation of spermatozoa and embryos, sperm sexing, and non-surgical embryo transfer. The following review will give emphasis to recent advancements in these reproductive technologies that are starting to show possibilities of serious applications under field conditions.

Introduction
Considerable interest has been directed during recent years toward the development and/or the improvement of emerging reproductive technologies to increase the efficiency of production in swine. This review describes several emerging reproductive technologies with potential application under field conditions in a short period of time, including artificial insemination (AI) with low number of spermatozoa, cryopreservation of spermatozoa and embryos, sperm sexing, and non-surgical embryo transfer (nsET). Although other reproductive biotechnologies, such as in vitro production of embryos, nuclear transfer and transgenic technology, have been successfully attempted, the low level of efficiency reached currently limits their use in applied production system.

Currently, different procedures for inseminating with a low number of spermatozoa in comparison with standard AI technique are commercially available. These procedures allow deposition of the sperm dose into the uterine body or in the depth of a uterine horn. With the use of deep intrauterine insemination (DUI), a 20-fold reduction in the number of fresh spermatozoa (Martinez et al. 2002) or a sixfold reduction in the number of frozen/thawed spermatozoa (Roca et al. 2003), can be achieved with reproductive performance results very close to those obtained after standard AI.

Sperm sexing technology is possible by using flow cytometric sorting of spermatozoa bearing X and Y chromosomes (reviewed in Johnson et al. 2005). Although first studies have reported satisfactory success rates following DUI with only 50-70 million sorted spermatozoa (Vazquez et al. 2003; Grossfeld et al. 2005), and the preferential birth of female using both technologies (Rath et al. 2003; Grossfeld et al. 2005), the number of spermatozoa required is still too large to be considered for practical application. An alternative method by using laparoscopic inseminations into the oviducts, where very high fertilization rates can be achieved using a number of flow-sorted spermatozoa as low as 300 000 (unpublished data), might be considered to be applied in high genetic value pigs.

Considerable advancements have been made in cryopreservation of embryos mainly by the open pulled straw (OPS) vitrification method. Currently a high percentage of embryos survive vitrification and warming procedures, indicating that this technology is effective for the cryopreservation of porcine embryos. In a related area, a new instrument for non-surgical deposition of embryos deep into the uterine horn of gilts and sows at days 4–6 of the oestrous cycle without sedation of the animals has been developed (Martinez et al. 2004). In the first attempts of non-surgical deep intrauterine transfer using fresh embryos, an acceptable reproductive performance (71.4% farrowing rate and 6.9 piglets born) was achieved (Martinez et al. 2004). Additionally, the first litters born after non-surgical deep intrauterine transfer of vitrified-warming embryos have been obtained (Cuello et al. 2005). Probably, a simple, effective and practical procedure for nsET in pigs will be available in the next few years, and together with OPS vitrification methods, important advances in the commercial applications of this technology will be achieved.

The developments of these technologies will be reviewed with emphasis on our own research.

Artificial Insemination
Artificial insemination is of great value for the pig industry. In the past 15 years there has been an enormous increase in the development of field AI services in the majority of countries. This increase in pig AI is because of increased demand for higher quality pork, and to increased profitability from high quality carcass together with improved aspects of the AI technique, mainly the development of several extenders for short or long-term storage of sperm (reviewed by Levis 2000). Furthermore, substantial advances have been made in other associated AI areas. Numerous investigations have been carried out to develop different tests for evaluating semen quality and functionality and predicting in vivo boar fertility (reviewed in Colebrander et al. 2000; Rodriguez-Martinez 2001, 2003; Gadea et al. 2005), although none of them are routine procedures in AI centres. Other investigations have been focused on the effect of time of insemination relative to ovulation, the role of seminal plasma in sperm transport and on the time of ovulation, and the addition of different compounds to the insemination doses to
enhance reproductive performance (reviewed in Waberski 1997; Soede et al. 2000; Langendijk et al. 2005). Recently, new investigations have been performed about the place of deposition of the insemination dose and the number of spermatozoa per dose. Current procedures of pig AI usually employ 2.5–4 billion spermatozoa per insemination in a large volume of liquid (70–100 ml) deposited intracervically two or three times during the oestrous period. These conditions limit the number of doses that can be prepared from one ejaculate to approximately 20–25. To increase the efficient use of spermatozoa of boars of high genetic merit, several strategies have been proposed, including to reduce the number of inseminations per oestrus to one insemination only (increasing the fertile life span of sperm cells or improving the prediction of ovulation time) (Soede et al. 2000), or to utilize new AI techniques, which allow a change in the site of semen deposition. It is known that a 100-fold reduction of the standard AI dose can be made when spermatozoa are surgically deposited close to the uterotubal junction (Krueger et al. 1999; Krueger and Rath 2000). Therefore, a practical method to reduce the number of spermatozoa per dose is to use intrauterine insemination. In this way, new procedures for depositing spermatozoa into the uterine body (postcervical insemination; Watson and Behan 2002) or into the uterine horn (DUI; Vazquez et al. 1999; Martinez et al. 2001a, 2002) have been recently described.

Post-cervical insemination
Several different instruments have been developed to transverse the cervix and deposit the inseminate in the uterine body or posterior horn of multiparous sows. The aim of these devices is to reduce sperm numbers necessary to achieve farrowing rate and litter size similar to that when standard AI is used. Watson and Behan (2002) reported a large field trial, conducted under commercial conditions, designed to test the effectiveness of this type of insemination. The standard AI method produced farrowing rates of 91.1, 91.8 and 65.8% whereas the post-cervical insemination method gave rates of 90.5, 90.5 and 86.9, for insemination with 3, 2 and 1 billion spermatozoa, respectively. Similarly, the mean litter sizes with the standard AI were 12.5, 12.6 and 10.6 and with the post-cervical method 12.3, 12.3 and 12.1, respectively. Only the 1 billion dose with the standard AI method offered a significantly (p < 0.001) lower farrowing rate and litter size. The authors concluded that post-cervical insemination in the sow is simple, effective and safe, and allows the sperm dose to be reduced to 1 billion spermatozoa. However, those results from that study do not necessarily reflect the data from similar procedures on other farms. Recently, it has been reported similar farrowing rates when post-cervical (1 billion spermatozoa) and standard (4 billion spermatozoa) AI methods are used (Rozeboom et al. 2004). In contrast, total pigs born was markedly lower (1.5 piglets less) in the post-cervical treatment compared with standard AI system. Similar results were reported by Levis et al. 2002. Furthermore, when the number of spermatozoa used for post-cervical insemination is reduced to 0.5 billion, the farrowing rate decreases approximately 10 points in comparison with standard AI group (78% vs. 88.2%, respectively) and the differences in litter size between both AI treatments are significantly amplified (9.4 vs 11.6, respectively) (Rozeboom et al. 2004).

Nevertheless, based on these reports, it seems possible to use the post-cervical insemination technique with 1 billion spermatozoa and to achieve acceptable reproductive performance. This could be important under determined situations, although field reports from excellent managed herds that achieve a high farrowing rate and litter size using standard AI with only 1.5 billion spermatozoa per insemination are becoming more common.

Deep intrauterine insemination
Despite the complex anatomy of the cervix and uterine horn of the sow, a new device (flexible catheter) for successful non-surgical DUI in non-sedated sows has been reported (Martinez et al. 2001a, 2002). Using that device, sperm dose can be deposited 8–55 cm from the uterotubal junction (Martinez et al. 2002). Several experiments carried out in our laboratory indicate that in comparison with standard AI (3 billion spermatozoa in 80–100 ml), a 20-fold reduction in the number of spermatozoa inseminated, and at least a 8–20-fold reduction in the dose volume can be used without affecting farrowing rate and litter size using deep intrauterine technique in weaned sows with induced oestrus (Martinez et al. 2001a, 2002). In an additional experiment, it was demonstrated that when the sperm dose is deposited once deep into a uterine horn, spermatozoa are able to reach the contralateral oviduct and to fertilize a high proportion of oocytes (Martinez et al. 2002). We can now to confirm that using induced oestrus sows with a uterine horn sectioned via laparoscopy, spermatozoa are able to reach the contralateral oviduct and fertilize a large proportion of oocytes following two different pathways: transperitoneal and intrauterine. While the transperitoneal pathway is effective in only a very small percentage of the sows (<5%), the intrauterine pathway seems be the predominant route (>75% of the sows) (unpublished data).

Reasons why the number of spermatozoa per insemination dose can be decreased when insemination is performed deep in the uterine horn are not clear. The possible mechanisms implicated in the effectiveness of DUI, including the physiological aspects of sperm transportation in the genital tract of the sow, have been recently reviewed (Martinez et al. 2001b; Rath 2002; Rodriguez-Martinez et al. 2005).

The possibility to carry out DUI in spontaneous postweaning oestrus sows would allow the application of this new technology under the same conditions that the conventional procedures of AI are used in commercial pig units. Several studies have been conducted in order to evaluate reproductive performance when this technique is used in non-induced post-weaning oestrus sows (Vazquez et al. 2001; Day et al. 2003; Martinez et al. 2005). In the USA, Day et al. (2003) used a total of 105 weaned sows, which were divided into two treatment
groups. One group was inseminated with 150 million sperm in 5 ml semen extender using DUI technique. This dose of semen was taken directly from the same batch of semen used to inseminate control weaned sows following standard AI procedure for the farm. Sows were inseminated two to three times per oestrus. Pregnancy and farrowing rates did not differ between DUI and standard AI groups. However, differences (p < 0.01) were found in the number of total piglets born (10.5 ± 0.49 vs. 12.9 ± 0.49, respectively) and born alive (9.0 ± 0.51 vs. 11.2 ± 0.50, respectively) between groups. In Spain, we have obtained similar results (Vazquez et al. 2001; Martinez et al. 2005). In those studies, using the same groups for insemination as described above, there was no significant difference in the farrowing rate between groups but the litter sizes resulting from DUI were approximately 1.0 piglet less than in standard AI groups. From a commercial point of view, the loss of 1–2 piglets per sow when DUI technique is used under field conditions is economically unacceptable. The question now is: why is the litter size lower when DUI with 150 million spermatozoa is used in sows at spontaneous oestrus? Various experiments are underway to determine the possible factors implicated in such reduction of prolificacy. Recently, we have finished an experiment using 71 sows with spontaneous post-weaning oestrus (Martinez et al. 2005). Fifty-five sows were deep intrauterine inseminated three times (12, 24 and 36 h after onset of oestrus) with 150 (n = 17), 300 (n = 19) or 600 (n = 19) million spermatozoa in 5, 10 or 20 mL BTS, respectively. The remaining sows (n = 16) were traditionally inseminated. On day 6 after oestrus, sows were subjected to laparotomy and the tips of both uterine horns were flushed in order to evaluate pregnancy rate (percentage of sows with at least four viable embryos) and fertilization rate (ratio of viable embryos to the total number of embryos and oocytes). Pregnancy rate was similar in all the groups. Fertilization rate and the percentage of bilateral fertilization after DUI with 600 million spermatozoa did not differ from that of the standard AI group (97.8 and 100% vs. 98.5 and 100%, respectively), but a significant decrease in both parameters (p < 0.05) was observed in sows inseminated with 300 (94.3 and 87.5%) or 150 (84.4 and 66.7%) million spermatozoa. The percentage of sows with different fertilization rates for each insemination group is shown in Fig. 1. Based on those results, we could conclude that the lower litter size achieved using DUI with 150 million spermatozoa under field conditions is related to the presence of approximately 33% of partial bilateral (when more than 50% of the structures collected from a uterine horn are oocytes) or unilateral fertilizations (when only oocytes are collected from a uterine horn) observed in that group.

In contrast to these results, as mentioned above, the litter sizes obtained in our first experiments (Martinez et al. 2001a, 2002) after a single deep insemination in one uterine horn of hormonally induced oestrus sows were not significantly different from those of the standard AI group (spontaneous oestrus sows). Besides, in an additional experiment (Martinez et al. 2002) using hormonally induced oestrus sows, the proportion of embryos collected 5 days after a single DUI with 150 million spermatozoa was similar in both uterine horns in the sows used for this purpose and no unilateral fertilizations were observed. Unfortunately, the number of sows used in this experiment was low (n = 5). Perhaps, if the number of sows used had been higher, unilateral and/or partial bilateral fertilizations might have been detected. The differences observed in the incidence of unilateral fertilization and in the litter size using DUI with 150 million spermatozoa in induced or spontaneous oestrus sows could be related to the insemination–ovulation interval. We can assume that when sows with induced oestrus were used, DUIs were performed within optimal insemination–ovulation interval to achieve the best fertilization rates. Furthermore, the ovariolytic treatment to the hormonal treatment also needs to be considered. It is possible that the hormonal treatment of sows inseminated in the depth of one uterine horn with 150 million spermatozoa increased the number of ovulations in each ovary and, as a result, a greater number of oocytes could have been fertilized in relation to standard AI sows (spontaneous oestrus sows), even with the presence of unilateral or partial bilateral fertilization. Consequently, the litter sizes were similar between both groups of sows.

Even with the current results, DUI might be of great benefit to pig industry, because the number of seminal doses per year obtained from a boar could be enormously increased. Other biotechnologies such as frozen semen, flow-sorted sperm or nsET could benefit from using this technology.

**Frozen Semen**

In spite of the potential applications of frozen-thawed spermatozoa in the pig industry, it is not generally used for AI programmes on pig farms because it is still unprofitable in comparison with the use of fresh or short-stored semen. The low reproductive performance achieved with frozen semen is well documented (Johnson et al. 2000). In comparison with fresh semen, the fertility obtained with frozen semen is lower and the litter size is approximately 2–3 piglets less. It is known that boar spermatozoa are more susceptible to cold shock than those of other species and that an important proportion of spermatozoa die during the freezing procedure.
In addition, a high proportion of motile spermatozoa post-thaw has decreased or suppressed fertilizing ability because the freezing-thawing causes destabilization of the sperm membrane (Watson 1996). In order to overcome these problems, and to increase the reproductive performance using frozen sperm, the current procedure for AI involves the deposition of a high number of thawed spermatozoa (5–6 billion) extended in 80–100 ml of diluent into the cervix. From the first protocols used (Pursel and Johnson 1975; Westendorf et al. 1975), numerous papers have been published in which several steps of the cryopreservation process have been evaluated (review in Johnson et al. 2000; Watson 2000). Recently, increased fertility rates have been reported using spermatozoa frozen in a new flat plastic package of 5 ml (Eriksson et al. 2001), or in 0.5 ml straws (Bussiere et al. 2000). Nevertheless, the fertility and prolificacy are still lower than the levels expected with fresh semen and standard AI. Currently, the development of DUI technique has lowered the number of frozen-thawed spermatozoa needed for successful inseminations (up to 1 billion), which improve the output of this technology (Tables 1 and 2; Roca et al. 2003). From results of that study, two conclusions can be drawn. Firstly, using DUI, the number of thawed spermatozoa can be decreased from the 5-6 billion used in standard AI to 1 billion without altering reproductive performance. This technique could overcome one of the limitations for commercial use of frozen semen because it would allow for a major improvement in the utilization of boars with respect to production of frozen sperm doses. Secondly, a lower farrowing rates and litter size compared with those obtained with fresh semen should be expected when spontaneous oestrus sows are deep intrauterine inseminated with 1 billion thawed spermatozoa. The lower fertility of frozen-thawed spermatozoa could be attributed to the fact that some spontaneous oestrus sows were inseminated outside of the optimal insemination–ovulation period. Because thawed spermatozoa have a limited life span in the female genital tract, their use reduces the optimal insemination–ovulation interval to a very short period of time (0–4 h before ovulation; Waberski et al. 1994). In this way, it has been demonstrated in spontaneous oestrus sows that when DUI with 1 billion spermatozoa is performed around the time of ovulation (checked by ultrasonography), the reproductive performance is significantly higher (farrowing rate 81.35% and 9.6 piglets) than when inseminations were carried out before (38.3% and 9.3 piglets) or after (25% and 7.0 piglets) ovulation (Roca et al. 2002). On the other hand, at the moment, the duration of oestrus seems to be the best estimator available for the time of ovulation because ovulation takes place at a relatively fixed two-thirds of oestrus (Soede et al. 2000). Although the duration of oestrus is highly consistent within a farm, it varies considerably among farms (Steverink et al. 1999). This fact might indicate that the time of ovulation in relation to the onset of oestrus is different among farms and that the optimal time of insemination should be modified on each farm. Recently, we have conducted a study to evaluate whether the interval between DUI with thawed semen and spontaneous ovulation can explain differences reported in the reproductive performance between farms (Bolarin et al. 2005). In that study, in Farm A a significantly higher proportion of sows (72.2%) were inseminated around of the time of ovulation than in Farm B (29.3%) (Table 3). Although the farrowing rates were similar when inseminated at ovulation on both farms (82.9 and 83.3%; Table 4), differences were obtained in the total farrowing rate between farms (75.3% vs. 57.3% in farms A and B, respectively; Table 4) because the number of pre-ovulatory and post-ovulatory sows inseminated in Farm B was higher (Table 3). Those results indicate that differences among farms in farrowing rates when frozen thawed semen is used might be due, in part, to ovarian status dissimilarities at the moment of insemination. Those

<table>
<thead>
<tr>
<th>Number of sows inseminated</th>
<th>40</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sows pregnant at 28 days (%)</td>
<td>29 (72.5)</td>
<td>32 (84.2)</td>
</tr>
<tr>
<td>Number of sows farrowing (%)</td>
<td>28 (70)</td>
<td>32 (84.2)</td>
</tr>
<tr>
<td>Number of piglets born (mean ± SEM)</td>
<td>259 (9.25 ± 0.23)</td>
<td>316 (9.88 ± 0.21)</td>
</tr>
</tbody>
</table>

Values, within rows, with different superscripts are significantly different (P < 0.05).

**Table 2.** Fertilization and farrowing rates and litter size of weaned sows after deep intrauterine insemination twice with a low number of fresh (150 million) or frozen-thawed (1 billion) spermatozoa (from Roca et al. 2003)

<table>
<thead>
<tr>
<th>Farm</th>
<th>Sows (n)</th>
<th>Pre-ovulatory (F)</th>
<th>Peri-ovulatory (O)</th>
<th>Post-ovulatory (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>97</td>
<td>17 (17.52)^a,b</td>
<td>70 (72.16)^a,b</td>
<td>10 (10.31)^a,b</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>33 (40.24)^b</td>
<td>24 (29.27)^b</td>
<td>25 (30.49)^b</td>
</tr>
</tbody>
</table>

*At insemination time, both ovaries were checked for ovulation by transrectal ultrasonography and sows were classified into three groups: F sows (follicles visible during the two examinations), O sows (ovulation visible during at least one examination) and C sows (corpora lutea visible during both examinations).

^a,bValues with different superscript in the same row are significantly different (p < 0.05).

^abValues with different superscript in the same column are significantly different (p < 0.05).

---

**Table 1.** Fertilization and farrowing rates and litter size of weaned hormonally treated sows inseminated once with deep intrauterine procedure using a low number of fresh (150 million) or frozen-thawed (1 billion) spermatozoa, and conventional insemination using thawed (6 billion) spermatozoa (from Roca et al. 2003)

<table>
<thead>
<tr>
<th>Farm Sows (n)</th>
<th>Pre-ovulatory (F)</th>
<th>Peri-ovulatory (O)</th>
<th>Post-ovulatory (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>97</td>
<td>17 (17.52)^a,b</td>
<td>70 (72.16)^a,b</td>
</tr>
<tr>
<td>B</td>
<td>82</td>
<td>33 (40.24)^b</td>
<td>24 (29.27)^b</td>
</tr>
</tbody>
</table>

*At insemination time, both ovaries were checked for ovulation by transrectal ultrasonography and sows were classified into three groups: F sows (follicles visible during the two examinations), O sows (ovulation visible during at least one examination) and C sows (corpora lutea visible during both examinations).

Values with different superscript in the same row are significantly different (p < 0.05).

Values with different superscript in the same column are significantly different (p < 0.05).
differences are likely associated with incorrect oestrous detection or with an intrinsic variability in the time of ovulation among farms.

Sexed Semen

The development of semen sexing is widely accepted as a major advance in reproductive technology. The possibility of pre-selecting the sex of the offspring can be very important for improving reproductive management in pig production, as well as allowing for the production of male and female crossbred lines. In addition, the discussion about the banning of the castration in male piglets is a current topic in Europe. Female production through sex pre-selection would benefit from sex pre-selection by allowing for the production of male and female crossbred lines. In this way, laparoscopic insemination is a more efficient technique in the insemination of sex-sorted boar spermatozoa, allowing pregnancies up to 80% when only 300,000 sorted spermatozoa are inseminated per hour (Johnson and Welch 1999; Johnson et al. 2005). However, the number of spermatozoa required to date is similar to other alternatives. However, the application of this technology depends on the number of sexed sperm produced per unit of time and the fertilizing ability, the number of spermatozoa required by insemination and the percentage of piglets with the expected sex. High-speed cell sorters produce until 15 million sorted spermatozoa per hour (Johnson et al. 2005). Therefore, there is an acute necessity to deposit the spermatozoa as close as possible to both the site of fertilization, and time of ovulation to achieve acceptable fertility. Deep intrauterine insemination technique, depositing 50–70 million spermatozoa in the anterior third of the uterine horn, in combination with the hormonal control of ovulation, have been shown as useful tools in the insemination of sex-sorted spermatozoa in pigs (Rath et al. 2003; Vazquez et al. 2003; Grossfeld et al. 2005). However, the number of spermatozoa required is still too large to be considered for practical application. As deeper insemination is performed, the number of inseminated spermatozoa could be lowered, allowing more sows to be inseminated. In this way, laparoscopic insemination is a more efficient technique in the insemination of sex-sorted boar spermatozoa, allowing pregnancies up to 80% when only 300,000 sorted spermatozoa are inseminated per oviduct (unpublished data). Those data provide hope that this technology could be applied in the swine industry, at least under specialized production management situations.

Embryo Transfer

The swine industry has a considerable interest for the use of embryo transfer (ET). The use of this technology would allow the movement of genetic resources with enhanced animal welfare, minimal risk of disease transmission and reduced transportation costs, in comparison with transport of live animals. Embryo transfer is also essential for the application of other reproductive biotechnologies. However, the commercial application of embryo transfer in the pig has been traditionally limited because of the requirement of surgical transfer procedures and the difficulties for long-term storage of pig embryos. Recently, new perspectives for embryo transfer have arisen with the development of new procedures for embryo cryopreservation and non-surgical ET methods.

Cryopreservation of embryos

Although frozen-thawed porcine embryos have resulted in offspring following transfer to recipients (reviewed in Berthelot et al. 2003), the cryopreservation of pig embryos has largely been ineffective by their high sensitivity to chilling injury (Wilmut 1972; Polge et al. 1974). This fact has limited their ability to be cryopreserved by conventional slow freezing methods. At present, vitrification is regarded as an alternative to traditional slow freezing procedures. Vitrification en-
ables rapid cooling of embryos by direct plunging in liquid nitrogen without ice forming even in the extracellular medium, because of a very high concentration of cryoprotectant (Rall and Fahy 1985). The development of vitrification systems has resulted in piglets born after transfer of pre-treated (delipidated or cytochalasin treated) or untreated vitrified/warmed porcine embryos in several laboratories (Kobayashi et al. 1998; Beebe et al. 2000; Berthelot et al. 2000, 2001; Cameron et al. 2000, 2004; Dobrinsky et al. 2000; Dobrinsky 2001).

Until recently, embryos from several species have been vitrified in straws of 0.25 ml volume; these straws limit the cooling rate to less than 2500°C/min (Rall 1987). Cooling rate seems to be a key factor in embryo vitrification. An increase in cooling rate decreases chilling injury (Rall 1987; Dobrinsky and Johnson 1994) and may permit a reduction of cryoprotectant concentration (Vajta 2000).

The success of vitrification procedures has been enhanced by using new containers such as OPS (Vajta et al. 1997), which increase the cooling rate approximately eightfold in comparison with the 0.25 ml straws. More recently, the OPS cooling rate has been increased by using straws with a smaller inner diameter and wall thickness (superfine open pulled straws: SOPS; Isachenko et al. 2003), with cryoloops (Lane et al. 1999), or with the development of a new device (Vit-Master®; Minitub, Tiefenbach, Germany), whereby the liquid nitrogen temperature is reduced to −210°C by applying negative pressure. However, it has been shown that increasing the cooling rate above approximately 20 000°C/min, as occurs when the SOPS (Isachenko et al. 2001) or Vit-Master-SOPS (Arav et al. 2002) procedures are used, does not enhance the efficiency of in vitro development of morulae and blastocysts after warming. Nevertheless, procedures which increase cooling rates to above 20 000°C/min have the possible advantage that vitrification probably occurs at lower cryoprotectant concentrations. As cryoprotective solutions are toxic to embryos, reducing their concentration might be beneficial for their survival. Clearly, more research is needed to optimize the cryoprotectant concentration required in each of the vitrification systems used.

On the other hand, it has been demonstrated that the stage of embryonic development (Dobrinsky 2001) influences survival of pig embryos after vitrification. Peri-hatching blastocyst stage embryos are known to show the highest in vitro development after vitrification and warming. However, for hygiene reasons, the International Embryo Transfer Society restricts the collection, cryopreservation and transport of embryos to those with an intact zona pellucida (Stringfellow 1998). Therefore, morula and blastocyst stage embryos seem to be most suitable for commercial embryo transfer. Morulae and blastocyst embryos have higher lipid content when compared with peri-hatching blastocysts (Niimura and Ishida 1980), which is thought to be responsible for their increased chilling sensitivity. It has been reported that pre-treatments, such as delipidation and cytoskeletal stabilization and centrifugation, can improve the success of cryopreserving morulae and blastocysts. It has also been demonstrated that OPS vitrification results in high survival rates after warming using untreated morulae and blastocysts (reviewed in Berthelot et al. 2003). It is obvious that protocols that require special manipulation and those that implicate the breaking of the zona pellucida are less suitable for routine commercial purposes. Survival and hatching rates of untreated vitrified/warmed embryos currently obtained in our laboratory are shown in Fig. 2 (Cuello et al. 2004a). In addition, untreated embryos surviving vitrification/warming procedures show a low number of apoptotic cells and 80% of them displayed ultrastructural characteristics similar to fresh blastocysts (unpublished data).

Important improvements are being carried out from a practical point of view. As in other species, the conventional warming method has been simplified from three steps to one step (direct warming), with results similar as in vitro embryo survival rates (Cuello et al. 2004b). The birth of the first litters using this new procedure has been reported (Cuello et al. 2005). Direct warming procedure should be an ideal method for use in practice, and its application to vitrified porcine embryos may substantially simplify the transfer procedure by warming embryos directly in a syringe, which would be immediately connected to the embryo surgical or nonsurgical transfer catheter without any special manipulations. In summary, advances in vitrification technology are more frequent each day; currently a high percentage of embryos survive the vitrification and warming procedures, which indicate that this technology is effective for the cryopreservation of porcine embryos. However, further studies of the factors affecting embryo survival after vitrification (cryoprotectant toxicity, composition of the vitrification and warming solutions, cooling and warming rates in relation to cryoprotectant concentration, etc.) are required for widespread application of this technology.
Non-surgical embryo transfer

Although considerable interest has been expressed by the swine industry for an effective and practical procedure for ET, and although Polge and Day (1968) demonstrated that pregnancy could be established in pigs by a non-invasive procedure, nsET was considered as an impossible technique for many years. The cervical folds, the coiled nature of the uterine horns and the lack of effective instrumentation were the principal obstacles in the development of this technology (Day 2000). In the 1990s, new investigations to deposit embryos by using a non-surgical procedure were developed (reviewed in Hazeleger and Kemp 2001). Unfortunately, the majority of them have not been successful. At present, only one research group has performed nsET transfer directly into the uterine body or beginning of a uterine horn without sedation of the recipient sows (Hazeleger and Kemp 1994; Hazeleger et al. 2000). Hazeleger et al. (2000) obtained a pregnancy rate of 59% with 11 embryos present at day 35 of pregnancy after non-surgical deposition of blastocysts in 0.1 ml of medium. However, when recipient sows were permitted to become to farrow (Hazeleger and Kemp 1994), or when the same procedure for ET was used under field conditions (Ducro-Steverink et al. 2004), the farrowing rate and litter size was lower (33% and 41% farrowing rate and 6.7 and 7.2 total born piglets, respectively). Although the procedure used in these studies can be applied in practice, further improvements needed to be made to increase reproductive performance after nsET. It is known that under physiological conditions, once the embryos reach the uterus, they remain in the tip of the horn until Day 6, and then progress through the horn during days 7 and 8 (Dziuk 1985). Therefore, when embryos in morulae or blastocyst stages are used, it seems logical to consider that their deposition in the uterine body is less appropriate for deposition of embryos than the middle or last third of the uterine horn (Wallenhorst and Holtz 1999). However, with the earlier procedures for nsET, embryos could not be deposited further away from the uterine body or beginning of a uterine horn, and therefore it was not possible to know whether ET results could be increased by depositing the embryos in the middle or even farther up the uterine horn. Additionally, another limitation of the previous nsET systems is that only sows can be used as recipients because the cervix in gilts is tightly closed. It has been shown after surgical ET that the uterine body is too low for an extended use in pork production even when DUI methodology is used. Laparoscopic insemination into the oviduct, where the number of spermatozoa needed to achieve high fertilization rates is as low as 300 000, might be an alternative method for this purpose, at least when applied under specialized production management situations. Finally, embryo vitrification and nsET technologies are promising procedures to be used in combination for the introduction of new genetic material in a farm and for the maintenance of germplasm for the future.

Conclusions

Important progress has been made in several emerging reproductive technologies. As a consequence, the practical application of these technologies is inching closer everyday. Low dose post-cervical (1 billion fresh spermatozoa per dose) or deep intrauterine (150–300 million spermatozoa per dose) inseminations should be of great benefit in use of semen from superior boars, or in sanitary contingencies when the number of doses to be used is decreased. In addition, deep intrauterine technology may also be a practical method for obtaining satisfactory reproductive performance with a low number of frozen/thawed spermatozoa (1 billion per dose). Consequently, the use of frozen semen as an alternative to replace transportation of live animals, and as a means to store valuable genetic material, could be dramatically increased from a commercial point of view. The possibility of pre-selecting the sex of the offspring can be very important for the pig industry by allowing for the production of male and female crossbred lines. Currently, the number of available flow sorted spermatozoa is too low for an extended use in pork production even when DUI methodology is used. Laparoscopic insemination into the oviduct, where the number of spermatozoa needed to achieve high fertilization rates is as low as 300 000, might be an alternative method for this purpose, at least when applied under specialized production management situations. Finally, embryo vitrification and nsET technologies are promising procedures to be used in combination for the introduction of new genetic material in a farm and for the maintenance of germplasm for the future.

Acknowledgements

The authors are grateful to BN Day and TC McCauley for reading the manuscript critically. Financial support from CICYT (AGF98-0533; AGL2001-0471; AGL2004-07546); FEDER (1FD97-370); CDTI (B288/98;03.180; 03-402, 04-0231); INIA (RZ01-019), MEC of Spain (PR2000-179); Séneca Fund of Murcia (PB/74/FS/02); CARM (2103SU0040); Miller Fund of the University of Missouri (C-4-22481) and Monsanto Company (G-2929) projects in many of the studies reported is also gratefully acknowledged.
References


Levis DGS, Burroughs S, Williams S, 2002. Use of intrasomatic insemination of pigs: Pros, Cons and Economics. In:


Submitted: 01.02.2005
Author’s address (for correspondence): Dr EA Martinez, Department of Medicine and Animal Surgery, University of Murcia, Murcia 30071, Spain. E-mail: emilio@um.es