# Single fixed-time laparoscopic intrauterine insemination as a tool to obtain low-diversity porcine embryos

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**ABSTRACT**: Double fixed-time insemination after ovulation induction is commonly used in pigs to obtain *in vivo* produced embryos at defined stages of development for downstream biotechnological applications. However, variations in the time of ovulation and fertilisation of the ovulated oocytes by spermatozoa, mainly in one of the inseminations, can cause diversities in embryo development. The aim of the present study was to reduce embryo diversity and to achieve a 'uniform outcome' of porcine embryo stages using single laparoscopic fixed-time insemination (LIUI). Altogether, 48 puberal German Landrace gilts were included in the study. Estrus of gilts was synchronized by 15-day long altrenogest (Regumate®) feeding and follicle development was stimulated with 850 IU eCG 24 h after the final altrenogest application. Ovulation was induced with 500 IU hCG 80 h after eCG. LIUI was performed 31 h after hCG treatment. Gilts under general anaesthesia were fixed in a dorsal position, a pneumoperitoneum was produced and three trocar cannulas were inserted into the abdomen for optics and instruments. Each uterine horn was carefully punctured 10–15 cm caudal from the utero-tubal junction with a 2.5 mm trocar. A 2.2 mm catheter was inserted about 3 cm into the uterine lumen and 20 ml of extended fresh boar semen (32.2 × 106 sperm cells/ml) were injected. Embryos were surgically flushed from the genital tract two (Day 2) and three (Day 3) days after insemination. Altogether, 778 oocytes/embryos were recovered (recovery rate 68 ± 17%); 45 of 48 gilts (93.8%) revealed fertilisation and 76.1% of the recovered embryos ($n$ = 592) were at the 2- and 4-cell stage. On Day 2 ($n$ = 22 gilts), a higher percentage of gilts (72.7%, $P < 0.05$) displayed only 2-cell embryos compared with gilts which had 2- and 4-cell (22.7%), or only 4-cell embryos (4.6%). On Day 3 ($n$ = 23 gilts), the proportion of gilts with 2-cell, 2- and 4-cell, and only 4-cell embryos shifted to 4.3%, 0% and 95.7%, respectively ($P < 0.05$). The results of the present study demonstrate high rates of fertilisation and homogenously developed embryos after single fixed-time laparoscopic intrauterine insemination in gilts. Additionally, these results were achieved by inseminating a 60% lower number of sperm cells per insemination dose compared to usual doses used for transcervical insemination. In conclusion, LIUI can be recommended for the *in vivo* production of embryos in a homogeneous developmental stage, and also as an alternative method for low-dose insemination.

**Keywords**: laparoscopic insemination; embryo diversity; pig

**List of abbreviations**

**BW** = body weight, **CL** = corpus luteum, **eCG** = equine chorionic gonadotropin, **hCG** = human chorionic gonadotropin, **IU** = international unit, **LIUI** = laparoscopic fixed-time insemination

*In vivo*-derived embryos at a defined stage of development are often a necessary requirement for biotechnological applications and research. However, different developmental stages of the embryos (embryo diversity) at the time point of recovery remain an issue pigs (Soede et al. 1992; Soede and Kemp

1993; Cardenas and Pope 1993). Since double fixed-time insemination after ovulation induction is commonly used in pigs to produce embryos, biological variations in the time of ovulation and fertilisation of the ovulated oocytes by spermatozoa, mainly in one of the inseminations, can promote heterogeneity in embryo development. To diminish embryo diversity and to achieve a 'uniform outcome' of porcine embryo stages, single fixed-time insemination can be used. In particular, laparoscopic intrauterine insemination can be a method of choice. Laparoscopic (oviductal) insemination was first described by Morcrom and Dukelow (1980). The potential of laparoscopic intrauterine insemination (LIUI) has been demonstrated in sperm-mediated gene transfer (Fantinati et al. 2005) and evaluation of sperm migration (Brüssow et al. 2006, 2011). The aim of the present study was to analyse the fertilization efficiency and the developmental diversity of embryos after single fixed-time LIUI.

## MATERIAL AND METHODS

**Animals and experimental design**. All procedures involving animal handling and treatment were approved by the Committee for Animal Use and Care of the Agricultural Ministerial Department of Mecklenburg-Vorpommern, Germany.

Altogether, 48 puberal German Landrace gilts at the age of nine months and with mean body weights of 132 ± 7 kg were included in the study. Oestrus was synchronised in all gilts by 15-day long altrenogest feeding (16 mg altrenogest/day/gilt; Regumate®, Janssen-Cilag GmbH, Neuss, Germany). Twenty-four hours after the last Regumate® feeding (08:00 h), each animal received a single intramuscular injection of 850 IU equine chorionic gonadotropin (eCG; Pregmagon®, IDT Biologika, Dessau-Tornau, Germany). Ovulation was induced 80 h later by administration of 500 IU human chorionic gonadotropin (hCG; Ovogest®, Intervet, Unterschleißheim, Germany).

**Laparoscopic intrauterine insemination**. Laparoscopic intrauterine insemination was performed 31 h after hCG treatment with 20 ml/uterine horn of extended, fresh boar semen (32.2 × 106 sperm cells/ml; 65% motility). LIUI was carried out as described previously (Brüssow et al. 2006, 2011). Briefly, general anaesthesia in gilts was induced with ketamine (0.15 ml/kg BW, Ursotamin®, Serumwerk Dessau, Germany) and azaperone (0.03 ml/kg BW, Stresnil®, Elanco Animal Health, Bad Homburg, Germany) and animals were fixed in a dorsal position. A pneumoperitoneum with $CO_2$ was automatically produced (Endo Tech, Munich, Germany). Thereafter, three trocar cannulas (Storz, Tuttlingen, Germany) were inserted into the abdomen (Figure 1) for 0° optics (ETB, Berlin, Germany) and grasping forceps (NeoMed, Gutach/Bleibach, Germany). All laparoscopic handling was observed on a video monitoring system (NeoMed, Gutach/Bleibach, Germany).

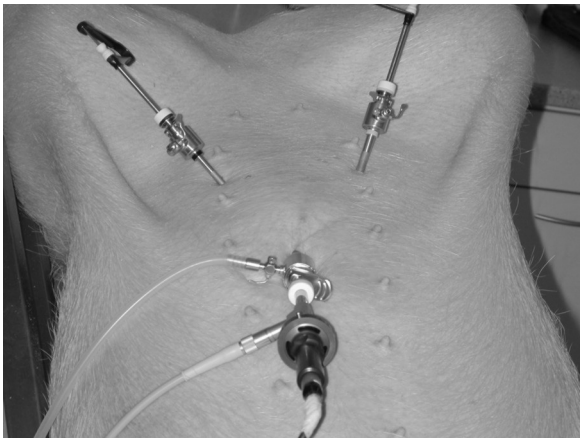

Figure 1. Three-point laparoscopy – trocar for 0° optics is inserted at the umbilicus; two trocars for the grasping forceps between the last two teats

For insemination, the uterine horn was carefully fixed with atraumatic forceps, and the uterine wall was punctured approximately 10 cm caudal from the uterotubal junction with a trocar, 2.5 mm in diameter (Figure 2). Under visual control, a 2.2-mm catheter (RÜSCH feeding tube, W. Rüsch AG,

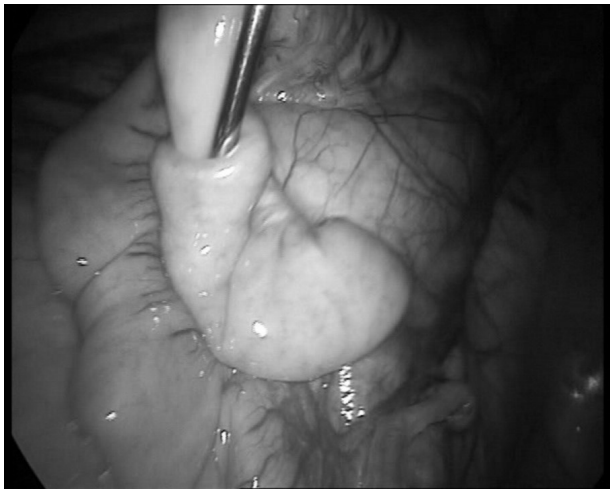

Figure 2. Puncturing of the uterine wall with a 2.5-mm trocar approximately 10 cm caudal from the uterotubal junction to introduce the catheter

Kernen, Germany) connected to a 20 ml syringe was inserted through the trocar cannula about 3 cm into the uterine lumen in the direction towards the tip of the uterine horn, and then semen was deposited. The insemination procedure was repeated at the opposite uterine horn and took about 15 to 20 min in total.

**Embryo recovery**. Embryos were surgically recovered on Day 2 or Day 3 after LIUI (i.e., 34–36 h or 58–60 h after insemination, respectively). The genital tract was exposed via midventral laparotomy under general anaesthesia as described above and the number of ovulation points (corpora haemorrhagica) per ovary was counted. Thereafter, the oviducts and about 10 cm of the adjacent uterine horns were surgically flushed with 20 ml Dulbecco's phosphate-buffered saline (DPBS, Lonza Walkersville, Walkersville MA, USA) supplemented with 0.3% BSA (Sigma, Taufkirchen, Germany). Ova were recovered from the flushing fluid under a stereoscopic microscope (Nikon SMZ800, Nikon, Japan) and immediately analysed for cleavage and cell number of the native embryo.

**Statistical analysis**. Calculation of means and their standard deviation and analyses of differences in mean values between groups was carried using the software package SigmaPlot 11.0. Differences between parameters with a normal distribution were tested by multiple pairwise comparison using the *t*-test or Mann-Whitney Rank Sum test if the test of normal distribution failed, respectively. Percentage distribution was analysed using the Chi-square test. Differences of $P < 0.05$ were considered significant.

## RESULTS

In all 48 gilts semen was successfully placed into the uterus using LIUI. Altogether, 778 oocytes/embryos were recovered from the genital tract of gilts (recovery rate: 68 ± 17%) and 45 of 48 gilts (93.8%) showed fertilization. The results of ovarian response and embryo development are presented in Tables 1 and 2, respectively.

No differences were obtained regarding the number of ovulations and the number of recovered oocytes and embryos in gilts on Day 2 and Day 3, respectively. Diverse stages of embryo development were observed when comparing embryos between the two time points of recovery, i.e., on Day 2 or Day 3. On Day 2, a higher percentage of gilts revealed 2-cell embryos compared to the other stages, and on Day 3 significantly more gilts had 4-cell embryos ($P < 0.05$).

## DISCUSSION

Embryo diversity is apparent in the pig and even synchronisation of ovulation does eliminate the presence of embryos at different stages of development (Nissen et al. 2000). After insemination of gilts twice at 27 h and 38 h and after ovulation induction

Table 1. Ovarian response and oocyte/embryo recovery in gilts on Day 2 and Day 3 after single fixed-time LIUI

| Group | Gilts (*n*) | *Corpus luteum* | | Oocytes | | Embryos | |
|---|---|---|---|---|---|---|---|
| | | *n* | means ± SD | *n* | means ± SD | *n* | means ± SD |
| Day 2 | 22 | 580 | 23.2 ± 9.2 | 420 | 16.8 ± 8.2 | 270 | 10.8 ± 8.8 |
| Day 3 | 23 | 571 | 24.8 ± 9.1 | 358 | 15.6 ± 5.9 | 322 | 14.0 ± 7.2 |

Table 2. Embryo development/diversity in gilts which revealed fertilisation after single fixed-time LIUI on Day 2 and Day 3

| Group | Gilts (*n*) | Gilts with | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2-cell embryos | | 2- and 4-cell embryos | | 4-cell embryos | |
| | | *n* | % | *n* | % | *n* | % |
| Day 2 | 22 | 16 | $72.7^{a,c}$ | 5 | $22.7^{b,c}$ | 1 | $4.6^{b,c}$ |
| Day 3 | 23 | 1 | $4.3^{a,d}$ | 0 | $0^{a,d}$ | 22 | $95.7^{b,d}$ |

Between embryo stages[a,b] $P < 0.05$; between days [c,d] $P < 0.05$

with hCG ova/embryos were surgically recovered (24 h after ovulation). Of these embryos 29% were at the 1-cell stage, and 66% and 5% developed to the 2- and 4-cell stage, respectively. Possibly, besides the variation in the time of ovulation, the fertilisation of the ovulated oocytes by spermatozoa of both inseminations could promote diversities in embryo development. Therefore, single fixed-time insemination could be used to moderate the diversity of embryos, since *in vivo*-obtained embryos at a defined stage of development are required for biotechnological applications. However, to our knowledge, data comparing embryo diversity after single and double insemination are missing. Application of spermatozoa close to the uterotubal junction to establish a sperm population in the isthmus reservoir that is sufficient to ensure optimal fertilisation would be beneficial, especially when low doses of semen are used. To this end, Martinez et al. (2001, 2002) carried out non-surgical deep intrauterine insemination via a flexible endoscope or catheter. However, a minimal-invasive approach using the laparoscopic technique is also reasonable. It also provides a controlled semen placement, but irritations of the endometrium by manipulating the endoscope in the uterus or by the use of a pneumouterus in a non-surgical deep intrauterine insemination procedure are minimised. Laparoscopic intrauterine insemination is successfully used in sheep for different purposes (Gourley and Riese 1990; Moses et al. 1997; Ehling et al. 2003; Pereyra-Bonnet et al. 2011). In pigs such a method has been used only to a limited extent so far (Fantinati et al. 2005; Brüssow et al. 2006, 2011).

Although laparoscopic intrauterine insemination requires specialised technical equipment and expertise, it is fast and minimal invasive. Furthermore, high fertilisation rates can be achieved. Fantinati et al. (2005) achieved fertilisation rates of 92.3 to 94.5% using 10 × 106 to 15 × 107 spermatozoa per uterine horn. Similarly, in our study 93.8% of gilts revealed successful fertilisation. Commonly, in routine artificial insemination 2 to 3 × 109 sperm cells per dose are inseminated (Krueger and Rath 2000). The insemination of sex-sorted semen or freeze-thawed semen, where the availability of vital spermatozoa is low, needs novel procedures for successful low-dose-insemination. The number of sperm cells can be reduced by applying the spermatozoa near to the tip of the uterine horn. Deep surgical intrauterine insemination with doses of 5 × 108 to 5 × 10 sperm cells yielded pregnancy rates of 64.3 to 100% when insemination was performed 32 h after ovulation induction with hCG (Krueger et al. 1999). In our study, the insemination dose per gilt was only 40% of the standard one and resulted in the fertilisation of 94% of gilts.

In the present study, embryo diversity could not be fully avoided using single fixed-time LIUI. However, the extent of diversity was diminished. On Day 2, the majority of gilts (72.2%) revealed embryos only at the 2-cell stage and the remaining gilts had embryos developed up to the 4-cell stage. On Day 3, 95.7% of the gilts displayed only 4-cell embryos. To our knowledge, there are no comparable data regarding attempts to diminish embryo diversity in pigs.

In summary, high rates of fertilisation and of relatively low-diversity developed embryos can be attained after single fixed-time laparoscopic intrauterine insemination in gilts. Using this method, the number of sperm cells per insemination dose can be reduced considerably. LIUI can be recommended not only for the production of embryos at defined developmental stages but also as an alternative for low-dose-insemination, which is needed for example for sex-sorted semen where the number of available sperm cells after the sorting procedure is low.

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