Original article

Laparoscopic embryo transfer in pigs – comparison of different variants and efficiencies of the method

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Abstract

The aim of the study was to develop a method of laparoscopic embryo transfer in pigs and to compare different variants of this method. Two catheter diameters (1.6 mm and 1.0 mm), the method and site of embryo deposition (oviduct or uterus), the embryo development stage (2 – 4 cell or blastocyst), the method for oviduct or uterus stabilization, the potential for cryopreserved embryo transfer, the developmental potential of the embryos after transfer to the oviduct, patomorphology of the oviduct after transfer and possible clinical complications were taken into consideration. Two studies compared two variants of transfer to the uterus, and five variants of transfer to the fallopian tube. The transfer of embryos by the infundibulum may be of limited use due to handling problems and very low efficiency (pregnancy was not achieved). Very low efficiency was shown after transfer of vitrified embryos. Transfer to the fallopian tube by puncture of the fallopian tube, regardless of the developmental stage of the embryo, is the recommended method of embryo transfer. The histopathological examination of the fallopian tube revealed possible changes within the puncture site. The numerous clinical complications observed did not affect the effectiveness of the method.

Keywords: laparoscopy, embryo, transfer, pig

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Introduction

Assisted reproductive technology (ART) methods are now often used and rapidly developing methods in veterinary medicine and reproductive biotechnology (Zeyland et al. 2014, Gadea et al. 2020). Embryo transfer (ET) is one of the critical stages of the ART procedure. Surgical, minimally invasive (laparoscopic) and non-invasive (through the uterine cervix) methods are used for embryo transfer. Surgical methods, although proven (Martinez et al. 2017) are not recommended. They are abandoned due to significant tissue injuries, numerous postoperative complications and. the long healing period. These methods have no public or legal acceptance in the light of existing rules on the protection of animals, maintenance of their welfare, and live animal studies. The alternative to surgical methods are minimally invasive or non-invasive methods (Martinez et al. 2013, Zheng et al. 2016, Brüssow et al. 2018). Minimally invasive methods use laparoscopic techniques and diminish surgical intervention while non-invasive (transcervical) methods do not require any surgical intervention (Hazeleger and Kemp 2001). Both of these methods saw extensive development in the 1990s (Besenfelder et al. 1997, Yongs 2001). In the last decade the focus is on improving noninvasive methods, and laparoscopic methods are given less attention. (Peltoniemi et al. 2019, Hiryama et al. 2020, Tajima et al. 2020). Non-surgical (transcervical) methods allow access to the end sections of the genital tract only, so their use is limited to the transfer of embryos to the uterus in the morula or blastocyst stage (Martinez et al. 2013). There are also some problems in determining the exact site of catheter insertion and embryo deposition (Martinez et al. 2013). Noninvasive methods cannot be used for the collection of oocytes and embryos, or not for the transfer of embryos to the fallopian tube. The collection of oocytes and embryos as well as the transfer of embryos in all developmental stages, from the zygote to the blastocyst, is possible only using to laparoscopic or surgery methods (Wieczorek et al. 2015, Tajima et al. 2020). The techniques of embryo transfer by laparoscopic methods in pigs differ, and the efficiency of transfer varies considerably from less than 20% to over 80% (Besenfelder et al. 1997, Hazeleger and Kemp 2001, Youngs 2001, Riha and Vejnar 2003, Wieczorek et al. 2015, Brüssow et al. 2018, Wieczorek et al. 2020b). It is influenced by many factors, including anatomical structure which makes manipulation within the uterus, fallopian tubes and uterine cavities very difficult, appropriate selection of donors, transfer technique, type of catheter used, developmental stage of embryos, place of embryo deposition, anatomy of the uterus and biotechnolo-gical processing e.g. vitrification. For example, embryos are transferred using different types of catheters e.g. catheters used in humans, Tomcat catheter, Foley catheter, or intravenous catheter (eg Venflon) or catheters developed by authors (Besenfelder et al. 1997, Hazeleger and Kemp 2001, Youngs 2001, Riha and Vejnar 2003, Wieczorek et al. 2015, Brüssow et al. 2018, Wieczorek et al. 2020b). Despite ongoing improvements, laparoscopic methods continue to be less efficient than surgical methods (Hazeleger and Kemp 2001). This provides motivation to improve the laparoscopic methods and to develop a simple and effective method with potential practical application. The aim of the present study was to compare the effectiveness of various variants of the method, taking into account critical factors such as: the place of embryo deposition, the developmental stage of embryos, diameter of the catheter, embryo vitrification, the developmental potential of the embryos after transfer to the oviduct, patomorphology of the oviduct after transfer and possible clinical complications.

Materials and Methods

All procedures were performed with the prior approval of II Animal Ethics Committee in Krakow, approval numbers 791/2010 and 582/2008.

Experimental layout

Seven variants of laparoscopic embryo transfer were used in two experiments. In the first stage, the method of embryo transfer into the uterus was investigated, in the second – the method of embryo transfer into the fallopian tube was examined. Two types of catheters differing in internal diameter (1.6 and 1.0 mm) were compared. In two of the variants (Variant 4 and Variant 5), the oviducts were subjected to histopathological examination following embryo transfer and the development potential of the transplanted embryos after 5-day incubation in the recipients' uterus was determined in vivo. The histological examination of the oviducts was performed to determine the invasiveness of the catheters and the possible impact on their functionality after the loss of oviductal wall integrity. The developmental potential of embryos after transplantation into the fallopian tube was determined. Blastocysts obtained after transfer of 2-4-cell embryos (variant 4 and variant 5) after 5-day culture in the oviduct and uterus were evaluated. Evaluation of the developmental potential of embryos in the remaining variants was not planned. The study was performed with a total of 236 Polish Landrace gilts (171 embryo donors and 65 recipients). These were

aged between 4 and 5 months (after first heat), clinically healthy, and derived from one herd.

All the embryos at the 2-4 blastomere stage were surgically recovered from the donors by flushing from the oviduct, according to the procedure described previously (Wieczorek et al. 2015). Cleavage embryos were selected for embryo transfer or for in vitro culture at 2-4 cells stage. Selected were those with equal-sized blastomeres with defined outlines, without or minimal (<10%) cytoplasmic fragmentation and without vacuoles in cytoplasm. Some of the embryos were transferred at the 2-4 blastomere stage within 1 h of recovery, and the others were cultured in vitro to the blastocyst stage. The obtained blastocysts were transferred into the oviduct or uterus and some of the blastocysts were vitrified; after warming, they were transferred into the oviduct and several were used for assessment of embryo quality. The embryos were transferred by laparoscopy.

Recipients and donors were synchronized for estrous cycle, and donors were additionally superovulated as it was described earlier (Wieczorek et al. 2015).

Culture and macroscopic examination of embryos

Embryos were cultured to the blastocyst stage for 5 days in North Carolina State University-23 (NCSU-23) medium (Peters and Wells 1993) in an incubator at 39°C in 5% CO_2. During culture, embryos were assessed at 24-h intervals under a stereomicroscope (100x magnification, Nikon SMZ-10A, Japan) in a laminar chamber (Thermo Scientific™, Heraguard™ ECO, Germany) at around 39°C. The obtained blastocysts were transferred into the oviduct or uterus. In addition, some of the blastocysts were used as a control group for the assessment of the quality by TUNEL assay. The following criteria were applied in the macroscopic examination of embryos: at an early stage of development, morphologically normal 2- to 4-cell embryos with the intact zona pellucida and a uniform cytoplasm were used; for embryos at a later stage of development, morphologically normal, large, oval and symmetrical blastocysts with a thin the zona pellucida and the distinct trophoblast were used. Only those meeting the standards of Grade 1 derived by The International Embryo Transfer Society (Van Soom and Boerjan 2012) were used.

Cryopreservation of blastocysts

Morphologically normal blastocysts were cryopreserved by vitrification according to a method reported by Gajda et al. (2004). A vitrification solution containing TCM-199 (Medium-199, Sigma-Aldrich) with the cryoprotective agents DMSO (Dimethyl Sulfoxide,

Sigma-Aldrich) and EG (Ethylene Glycol, Sigma-Aldrich) was used. Blastocysts were incubated for 3 minutes in a pre-vitrification solution containing 7.5% DMSO (DMSO, Sigma-Aldrich) and 7.5% EG (Ethylene Glycol, Sigma-Aldrich), and then transferred for 1 minute to the vitrification solution composed of 18% DMSO (DMSO, Sigma-Aldrich) and 18% EG (Ethylene Glycol, Sigma-Aldrich). Blastocysts were stored in a liquid nitrogen tank.

Embryo transfer

The same protocol to prepare an animal to the experiment and the anesthetic protocol was used in all the animals. A feeding was stopped 24 hours before the procedure. A free access to water was assured. Complex anesthesia was carried out through a premedication with atropine 0,06 mg/kg i.m. (Atropinum Sulfuricum 0.5 mg, Polfa) and azaperone 3 mg/kg i.m. (Stresnil, Janssen Animal Health BVBA). A caniulation of the ear marginal vein was performed (20G, Venflon BD 1.0). General infusion anesthesia was used with ketamine 10-15 mg/kg i.v. (Ketamina 10%, Biowet Pulawy, Poland) and ksylazine 2.0-3.0 mg/kg i.v. (Sedazin, Biowet Pulawy, Poland). A constant control of respiratory and circulatory systems was performed. After insertion of the first trocar and the insertion of the camera, the abdominal cavity was filled with filtered air for organ visualization and manipulation. After visual inspection of the abdominal cavity, another 2 trocars were placed and the grips were introduced. In the generally laparoscopic transfer scheme an endoscope camera was positioned midway between teat pairs 2 and 3 or in the navel, the left grasper between left teat pairs 4 and 5 and the right grasper between right teat pairs 4 and 5. The catheter was inserted into the abdominal cavity at the height of teat pair 5 and 6 laterally from the midline (Fig. 1). Embryos were transferred into the uterus using a catheter of 1.6 mm internal diameter, and into the oviduct using 2 catheters – the uterus catheter (1.6-mm diameter) and the oviduct catheter (1.0-mm internal diameter). Deposition of embryos was via injection into the uterine or oviductal lumen. After deposition, the catheter, graspers and trocars were removed in reverse order to that in which they were inserted. After removing the trocars, single simple sutures were placed in the skin. The peritoneum and muscles, due to the minimal wound of 5-10 mm diameter, required no suturing. Pregnancies were diagnosed ultrasonically between 28 and 31 days after the procedure. Variants:

Variant 1. Into the uterus – stabilization of the horn at the mouth of the oviduct (in the initial, proximal part)

In this variant, the uterus was stabilized by grasping it as close to the beginning of the uterine horn and the
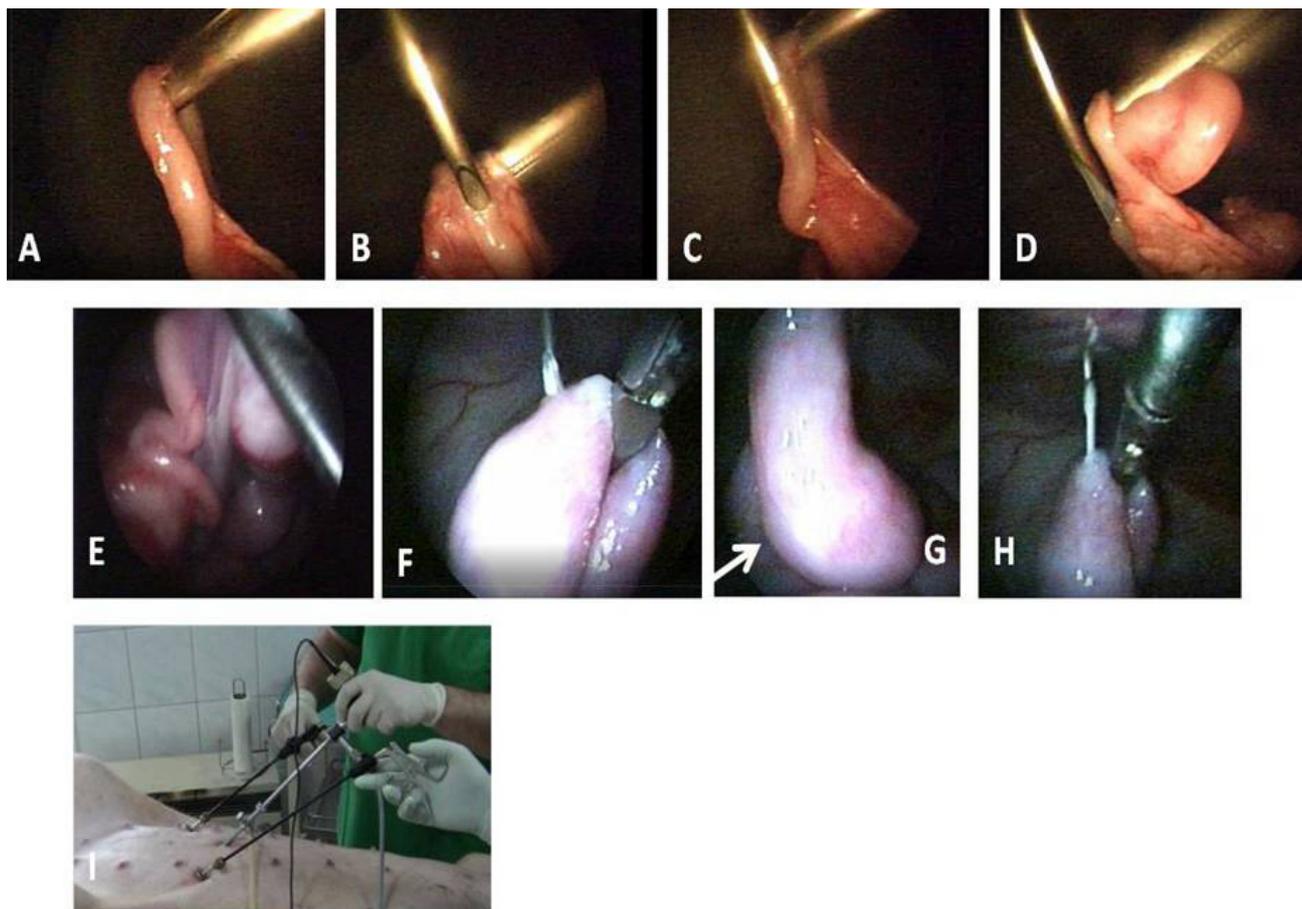
Fig. 1. Laparoscopic porcine embryo transfer. A - fallopian tube stabilization B - fallopian tube puncture; C - insertion of the needle into the lumen of the fallopian tube; D - removal of the needle and catheter from the fallopian tube; E - stabilization of the uterine horn at the mouth of the fallopian tube; F - uterine stabilization in 1/2 of the horn length, uterine puncture with a needle; G - the introduction of the cater into the lumen of the uterus; H - removal of the needle and catheter from the uterine horn; I - arrangement of trocars and manipulators.

fallopian tube outlet as possible. The catheter set for transplantation of porcine blastocysts into the uterus (Wieczorek et al. 2010) was used. Catheter of 1.6-mm internal diameter and of 1.8-mm external diameter to allow the passage of a standard 1.6 mm needle (16G), was punctured into the lumen of the uterus 3-5 cm deep. Blastocysts obtained in vitro were transferred to 15 recipients (*n*=15).

Variant 2. Into the uterus – stabilization of the horn at between ½ - 2/3 length (in middle part)

Following stabilization of the horn, the procedure was performed as in Variant 1, using the same set of catheters (Wieczorek et al. 2010). Blastocyst-stage embryos were transferred to eight recipients (*n*=8).

Variant 3. Into the oviduct through the infundibulum

Five trocars were inserted into the abdominal cavity, one for the endoscope camera and the other 4 for graspers. Four graspers were used to stabilize the infundibulum of the oviduct, to visualize the orifice of the oviduct, through which a 1.0-mm internal diameter catheter was inserted 2-3 cm deep. In this variant, em-

bryo transfer required the contribution of a third operator. Embryos at the 2- to 4-blastomere stage were transferred to 6 recipients (*n*=6). In this group, 6 out of 14 planned procedures were performed due to complications and low efficiency.

Variant 4. By puncture into the oviduct, with an intrauterine embryo transfer catheter

A catheter of 1.6-mm internal diameter (as in Variants 1 and 2) for transplantation of porcine blastocysts into the uterus was used (Wieczorek et al. 2010). In this group, 5 out of 14 planned procedures were performed due to complications and low efficiency. Embryos at the stage of 2-4 blastomeres were transplanted into 5 recipients, one of which was euthanized on the fifth day after embryo transfer to harvest the uterus and oviducts. Oviducts were submitted for histological examination, and blastocysts were flushed from the uterus for quality assessment using TUNEL assay.

Variant 5. By puncture of the oviduct, with an oviductal embryo transfer catheter – 2- to 4- blastomere embryos

In comparison to Variant 4, a original catheter with

greater flexibility and an internal diameter reduced to 1.0 mm and external to 1.2 mm was used for embryo transfer. The oviduct was punctured with a standard injection needle (1.2 mm; 18G, BD Microlance). A catheter with embryos was inserted 3-5 cm deep through the needle into the oviductal lumen. 2- to 4-blastomere embryos were transferred into 14 recipients, four of which were euthanized on the fifth day after embryo transfer to harvest the uteri and oviducts. The oviducts were submitted for histological examination. Blastocysts flushed from the uterus were evaluated by TUNEL assay.

Variant 6. Blastocyst transfer into oviduct

Following the procedure used in Variant 5, blastocyst-stage embryos after in vitro culture were transferred to the oviduct after 5-day culture in vitro. The patent "A method for transplantation of vitrified porcine blastocysts" was applied (Gajda et al. 2008). Ten recipients were used (*n*=10).

Variant 7. Into the oviduct – blastocysts following vitrification

Finally, cryopreserved blastocyst transplants were performed. Using the same procedure as in Variants 5 and 6, cryopreserved embryos at the blastocyst stage were transferred to the oviduct. Vitrified embryos were warmed and transferred by laparoscopy. Seven recipients were used (*n*=7).

Transplantation efficiency in all the variants was assessed based on the percentage of pregnant recipients (ultrasound examination between 28-31 day after procedure), number of piglets born, number of piglets born alive, and number of weaned piglets were taken into account. The results from the study groups were compared among the groups and also with the results obtained on the farm from which the pigs originated, following routine insemination (control group). As the control group there were 24 gilts, randomly selected from the same herd and with the same condition as the recipients and donors gilts.

Assessment of transferred embryos by TUNEL assay

On day 5 after the transfer, five recipients (Variant 4 *n*=1, Variant 5 *n*=4) were euthanized with intravenous sodium pentobarbital (50-75 mg/kg) (Morbital 100 ml, Biovet, Poland). Uteri with oviducts and ovaries were collected. After 5-day in vivo culture, transferred embryos (blastocysts) were flushed from the uterus. The control group were embryos obtained from donors and cultured in vitro as previously described. The qualitative analysis was performed via TUNEL assay with the use of an In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). The number of stained cells (nuclei) in blastocysts was counted under an Eclipse E600 epifluorescence microscope (Nikon, Japan) using a 358-461 nm filter, and the number of apoptotic nuclei was counted using a 520 nm filter. The apoptotic index was counted as the ratio of apoptotic nuclei to all nuclei.

Histological examination of oviducts following embryo transfer

One oviduct after treatment with a 1.6-mm catheter (Variant 4) and 4 oviducts after embryo transfer with a 1.0-mm catheter (Variant 5) were collected for the examination. The control group were 3 oviducts into which no embryos were transferred. The collected oviducts were fixed in 10% formalin (10% buffered formalin, Pol-Aura, Zabrze, Poland) and then cut into 2-3 mm transverse sections using a microtome knife. The sections were placed into labelled histology cassettes while observing the sequence along the oviduct, placed in an automated tissue processor (Shandon Excelsior ES, Astmoor, UK) and embedded in paraffin. Paraffin blocks were sectioned into 2- to 3-µm-thick sections, which were mounted in a glass slide and stained with hematoxylin-eosin in an automated slide stainer (Shandon Varistain Gemini, Astmoor, UK), and then placed under a coverslip and examined under a light microscope. Histological changes were graded on a 4-point scale: 0 – none, 1 – minor, 2 – moderate, 3 – considerable.

Clinical observations

Clinical observations of 65 recipients were made during the transplantation and up to 28-31 days after the procedure (up until the ultrasound examination for pregnancy). Non-pregnant gilts were excluded. All pregnant gilts were observed throughout the entire pregnancy until parturition, and then until weaning. The type and severity of complications during the procedure were noted. As in the histological examination, the intensity of complications was graded on a 4-point scale.

Statistical analysis

Statistical analysis was done using Statistica 13.3 software (Tibco Statistica, Kraków, Poland). One-way analysis of variance for independent samples was performed with the Anova Kruskal Wallis test, with a significance level of $p < 0.05$. The variables were treated as independent samples. The X^2 statistic was used to verify the hypotheses.

Table 1. Evaluation of the effectiveness of variants of the porcine embryo transplantation method based on the percentage of pregnant recipients.

Item	Experiment I Transfer embryo into uterine horn			Experiment II Transfer embryo into oviduct						Together (All recipients)	Control (Farm animals)
	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	Variant 6	Variant 7	Histology 1.6 catheter	Histology 1.0 catheter		
Recipients (No.)	15	8	6*	4*	10	10	7	1	4	65	24
Number of transferred embryos	547	266	171	121	312	313	236	20	60	2046	-
Number of transferred embryos / one recipient	36±5	33±2	28±1	30±0.5	31±2	31±3	33	20±0	15	31.5±6	-
Number of pregnant recipients (‰)	6 (40%) [A,B]	4 (50%) [A]	0 (0.0%)	1 (25%)[A]	6 (60%)[A,B,C]	4 (40%)[A,B,C]	1 (14%)[A]	-	-	22 (34%)[A]	20 (83%)[A]

A – statistically significant differences between variant and K p≤0.05; B – statistically significant differences between Variant 1 and Variant 5, C – statistically significant differences between Variant 5 and Variant 6; p≤0.05; * - 10 transplantations in the group were planned, the number was limited due to the observed complications

Results

Efficacy of different techniques of laparoscopic embryo transfer

In 171 embryo donors, a total of 2702 ovulations were observed and 2329 (86%) embryos at an early stage of development (2-4 blastomere stage) were harvested. Out of these embryos, 1555 were cultured in vitro, the rest 774 were transferred to recipients. The culture resulted in 1447 blastocysts, of which 575 were transferred to the uterus in Variant 1, 266 blastocysts to the uterus in Variant 2, 313 to the oviduct in Variant 6, and 293 were assigned to vitrification, from which 266 were vitrified following assessment. After warming of vitrified blastocysts, 236 were transferred to the oviduct (Variant 7). The other 816 embryos were transferred to the oviduct within 1h of recovery (Variants 3, 4 and 5). The embryos were transferred to a total of 65 recipients, 23 of which had embryos transferred to the uterus (Variants 1 and 2) and 42 to the oviduct (Variants 3, 4, 5, 6 and 7). An average of 31.5 embryos were transferred per recipient. The transfer of embryos resulted in 22 litters (all variants), 214 piglets born (196 piglets born alive, 92%) and 84% (179) of the piglets born were weaned. Seventeen piglets died before weaning, all of them within 5 days of birth (13 piglets died from acute diarrhea and 4 piglets were crushed by sows). The number of piglets born per litter averaged 10.2, and the number of piglets weaned per litter averaged 8.5 per pregnant sow.

Embryo transfer

The results are shown in Table 1.

Variants 1 and 2

After the embryos were transferred to the uterus, Variants 1 and 2 resulted in an efficiency of 40% and 50% respectively and the same number of piglets born per sow. In Variants 1 and 2, fewer piglets born alive per donor and fewer piglets weaned per donor were obtained compared to Variants 5, 6 and farm results.

Variants 3 to 7

In Variant 3, no pregnancy was obtained after the embryos were transferred into the oviduct via the infundibulum. This variant is very difficult manually and required the involvement of an additional operator and grasps. Transfer through the infundibulum allowed for shallow insertion of the catheter (to a maximum of 2-3 cm). In Variant 4, a low efficiency of 25% (1/4 recipients) was achieved after the embryos were transferred to the oviduct with an intrauterine embryo transfer catheter. Only 5 of the planned 14 transplants were performed in this group. After transfer of embryos to the fallopian tube, significant resistance during the insertion of the catheter and difficulties in inserting the catheter into the fallopian tube to the appropriate depth were observed in all recipients. In this group, manual difficulties of operators, low efficiency and severe injuries of the fallopian tube confirmed by histological and clinical examinations were observed. Therefore, after the first procedures, further transplants were
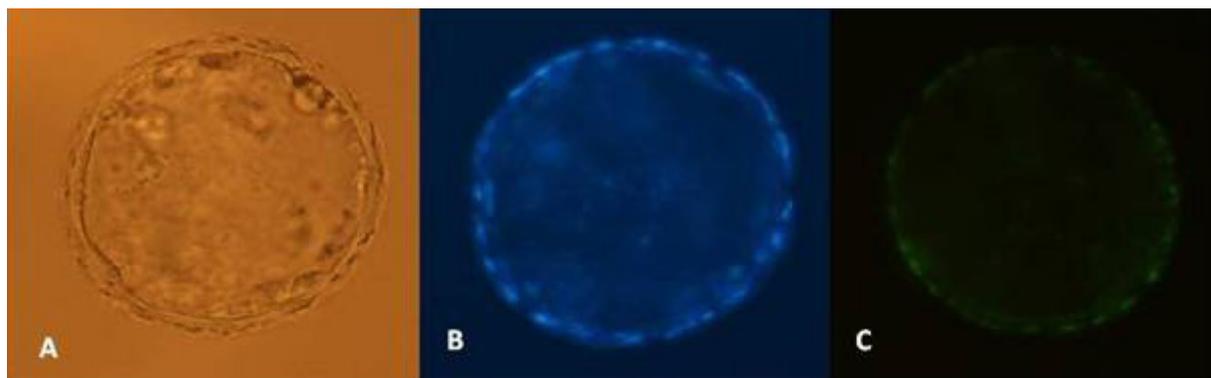
Fig. 2. A porcine blastocyst obtained from the uterus, after 5 days incubation in the uterus (A), stained by TUNEL and evaluated using a filter wavelength 358-461 nm (B,C) (x400).

Table 2. Assessment of the quality of expanding porcine blastocyst embryos after in vivo and in vitro culture based on the TUNEL test – the total number of nuclei and the number of nuclei showing apoptotic changes and the apoptotic index.

Item	Control (in vitro culture)	Group I (Blastocyst transferred 1.6 catheter after in vivo culture)	Group II (Blastocyst transferred 1.0 catheter after in vivo culture)
Number blastocysts	5	3	7
Total number cell nuclei / one blastocyst	58.00±6.82	60.00±8.66	54.86±2.41
Number of apoptotic nuclei / blastocyst	8.2±2.28	8.7±2.08	8.00±1.91
Apoptotic index (%)	14.5	14.7	14.5

No statistically significant differences between groups were found.

abandoned. After the embryos were transferred to the oviduct, the best results were obtained in Variant 5, the percentage of pregnant recipients was statistically higher than in Variants 1 and 6. After transferring blastocysts (Variant 6) into the oviduct, the same efficiency as after transferring blastocysts into the uterus at the mouth of the oviduct (in Variant 1) was detected and no statistically significant differences after transfer to the uterus at 1/3 - ½ of the horn length (in Variant 2) was obtained. In Variant 6, the pregnancy percentage was also significantly lower compared to the results for Variant 5 and farm results. In variant 7, the pregnancy rate was lower as well as the fetuses died and were resorbed. No piglets were obtained after transfer of vitrified blastocysts. In variants 1-6 numbers of piglets born per one sow were similar (no statistically significant differences). Live piglets born from one sow and piglets weaned from one sow in variants 4, 5 and 6 were higher compared to variants 1 and 2. Percentage of live born piglets and the percentage of weaned piglets were similar between the variants and compared to the control group (no statistically significant differences).

Assessment of transferred embryos by TUNEL assay

The results are shown in Table 2 and in Fig. 2. Eight donors produced 86 embryos, and after assessment, 80 embryos were transferred to 5 recipients (one in Variant 4 and four in Variant 5) and the other 6 embryos were retained for in vitro culture as a control group. Five days after the embryo transfer, of the 80 transferred embryos, 10 (12.5%) blastocysts were flushed (Variant 4 *n*=3, Variant 5 *n*=7). This method was also used to assess 6 blastocysts obtained in vitro. The developmental potential and quality of the embryos following in vivo culture and transfer with 1.6 catheter (Variant 4) and 1.0 catheters (Variant 5) were similar (no statistically significant differences between the groups). No differences were observed either in the number of nuclei and in the apoptotic index – both groups had a similar number of nuclei in blastocysts and a very similar apoptotic index. Also compared to the control group, after in vivo embryo culture, the number of nuclei in blastocysts and the apoptotic index were similar (no statistically significant differences between the groups).
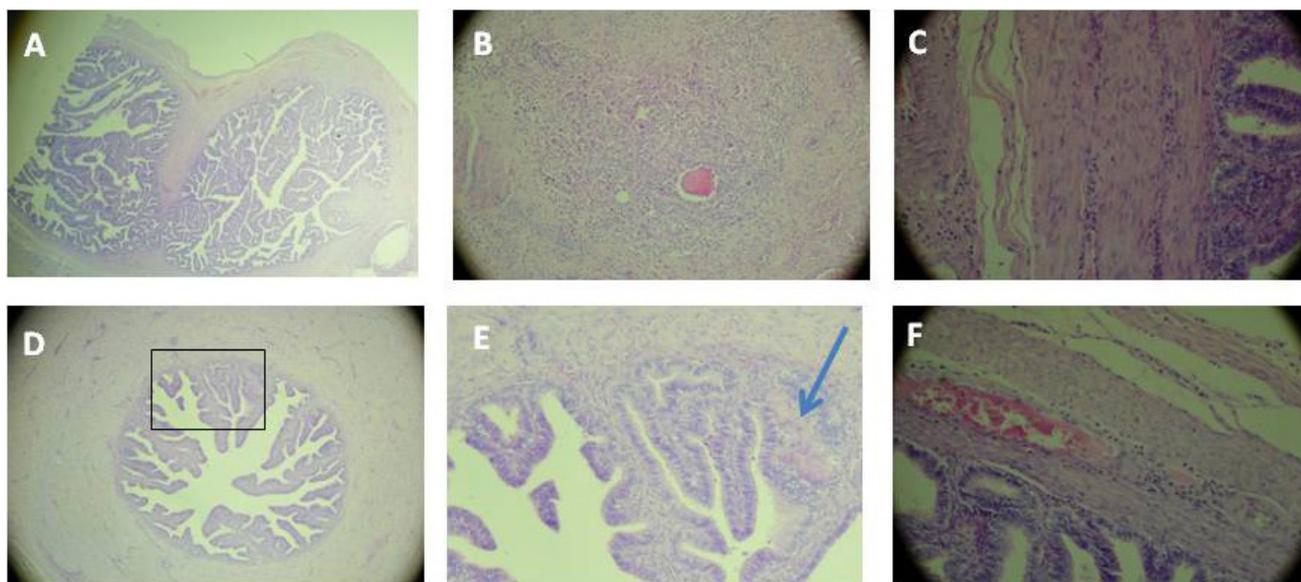
Fig. 3. The porcine fallopian tubes in histopathological examination. A – C the porcine oviduct after 1.6 catheter insertion: A (x25) - focus of acute inflammation with granulocytic infiltration around the circular defect, remnant of tubal puncture; B (x200) and C (x400) - concentrations of non-specific granulation tissue and mixed-cell inflammatory infiltrates in the fallopian tube wall; D – F – oviduct after 1.0 catheter insertion: small focal granulocytic infiltrates in the wall and lumen of the fallopian tube, D (x100); E (x200) - magnification of the frame in Fig. D, the arrow indicates clusters of granulocytes; F (x400).

Table 3. Histological evaluation of the fallopian tubes 5 days after porcine embryo transfer.

Item	Variant 4 (n=1)	Variant 5 (n=4)	Control (n=3)
Reduction in the lumen of the fallopian tube	3.0	0.0	0.0
Change in the structure of the mucosa	1.0	0.25±0.5	0.33±0,6
Fallopian tube wall defect after puncture	3.0	0.0	0.0
Inflammatory infiltrates around the injection	3.0	0.25±0.5	0.0
Granulocytic infiltration in the wall	3.0	0.5±0.6	0.33±0,6
Granulation tissue in the wall of the fallopian tube	3.0	0.0	0.0

There were no statistically significant differences. Statistical analysis was performed for Variant 5 and Control, Variant 4 was not analyzed.

Histological examination of oviducts

The results are presented in Table 3 and Fig. 3. In the fallopian tube to which the embryos were transferred using an intrauterine embryo transfer catheter (variant 4), only one examination was performed, where 5 extensive histopathological changes were found, which indicates a high invasiveness of the 1.6 mm catheter. Due to extensive complications, transplantations in this variant were suspended.

In the oviducts into which the embryos were transferred with a 1.0-mm catheter and in the control oviducts, out of the 6 examined characteristics there were 3 minor histopathological lesions in the form of structural changes of the mucous membrane, inflammatory infiltrations around the puncture site and granulocytic infiltrations (Table 3). For the described changes that occurred after puncture with a 1.0 catheter, no significant difference was noted in comparison to the control oviducts.

Clinical observations

The results are shown in Table 4 and in Fig. 4.

Bleeding from subcutaneous blood vessels after piercing the skin with a trocar as well as emphysema between the parietal and visceral peritoneum were most often observed. In addition, there was bleeding from the uterus at puncture site, but no bleeding from the oviducts occurred. In Variant 3, the integrity of the infundibulum was compromised by the graspers and the infundibulum was perforated by the catheter. In variant 4, during the insertion of the catheter, damage to the fallopian tube were observed in all recipients. In two cases, the large intestine was perforated during trocar insertion. After consulting a veterinarian, the animals with
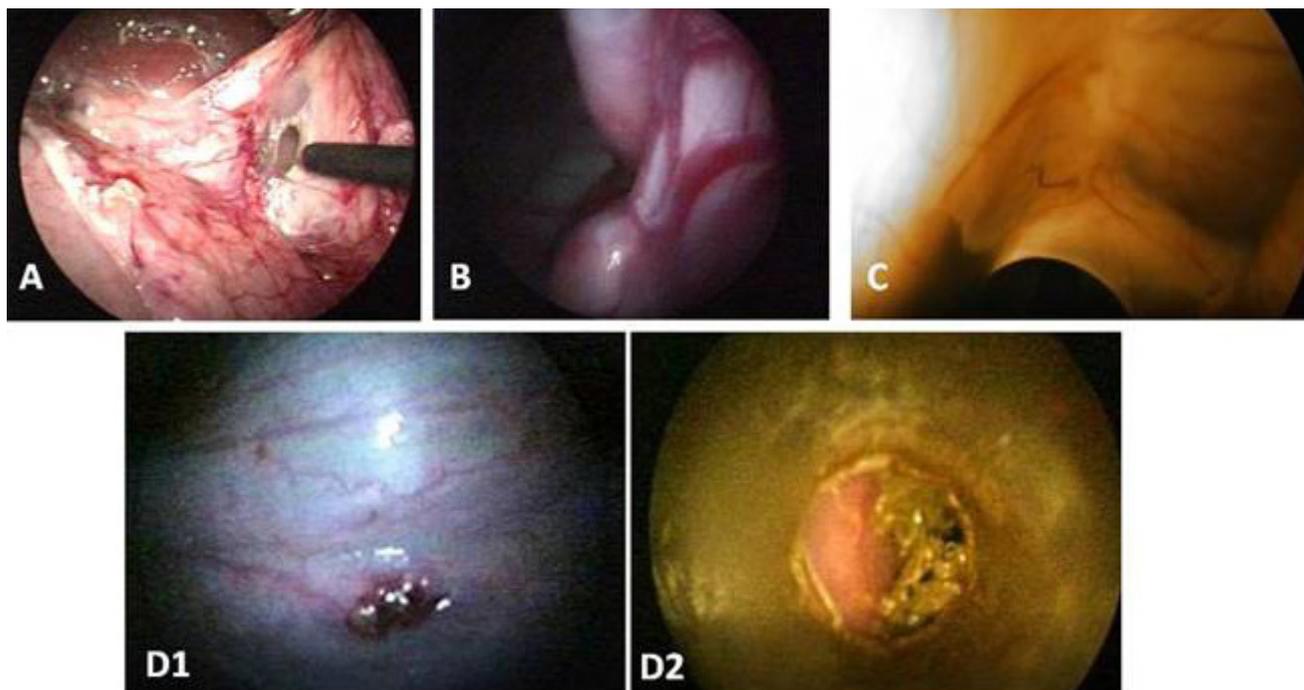
Fig. 4. Complications observed during laparoscopic embryo transfer in pigs. A – fallopian tube damage; B – bleeding after uterine puncture, C – pneumoperitoneum; D – puncture of the large intestine (D1 – the placet after trocar puncture, D2 - visible intestinal lumen).

Table 4. Clinical complications observed during the laparoscopic porcine embryo transfer procedure in all variants.

Item	Experiment I Transfer embryo into uterine horn			Experiment II Transfer embryo into oviduct				Total
	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5	Variant 6	Variant 7	
Pneumperitoneum	6	3	2	0	4	2	3	20
Bleeding from the subcutaneous vessels after insertion of the trocar	4	3	4	2	1	5	2	21
Bleeding after uterine puncture	6	4	0	0	0	0	0	10
Fallopian tube damage	0	0	0	4	0	0	0	4
Bleeding from the fallopian tube	0	0	0	0	0	0	0	0
Damage to the infundibulum of oviduct	0	0	4	0	0	0	0	4
Infundibulum of oviduct perforation	0	0	4	0	0	0	0	4
Puncture of the large intestine	1	0	0	0	1	0	0	2

perforated wall of the large intestine were closely observed for 21 days, subjected to 48-hour feed withdrawal, and their treatment was withheld until disease symptoms appeared. During the observations, no disease symptoms appeared after puncturing the large intestine, and after 21 days it was considered that the perforations healed spontaneously and had no effect on the trans-plantation process and efficiency. No differences were observed between the variants in the frequency and intensity of the complications. Parturitions were spontaneous, without assistance, and no deviations from the physiological norm were also observed until weaning.

Discussion

Laparoscopic methods of embryo transfer allow you to achieve 33% - 80% of pregnancy (Youngs 2001). In different described methods of laparoscopic embryos transfer to the abdominal cavity in various schemes, 3 to 5 trocars with a thickness of 5-10 mm are introduced (Besenfelder et al. 1997, Hazeleger and Kemp 2001, Youngs 2001, Riha and Vejnar 2003, Wieczorek et al. 2015, Brüssow et al. 2018, Wieczorek et al. 2020b). Our research uses the original catheter and a diagram of the introduction of endoscopic tools into the abdominal cavity (Wieczorek et al. 2020b). The efficiency of transfer to the uterus (variant I and II) was similar to that of other authors (Besenfelder et al. 1997, Wallenhorst and Holtz 1999, Riha and Vejnar 2003, Brüssow 2018). Transferring embryos into the oviduct with intrauterine catheters did not allow us to obtain high efficiency, and damage to the oviduct and other complication during puncture eliminated the use of a 1.6 mm catheter. Therefore, we developed an original two-part set of catheters for embryo deposition in the oviduct (Variant 5) and a high pregnancy rate was obtained. The catheter 1.0 mm has very good material properties, maintains geometric stability, smooth surface and features high tensile strength and adequate flexibility (Wieczorek et al. 2020b, 2022). The histological examination of the 1.0-mm catheter showed minimal trauma incidence. Changes in the histological examination of oviducts after transplantation with a 1.0-mm catheter and non-punctured oviducts were similar. A mild inflammatory infiltration was observed in the oviductal wall in both instances. Such small inflammatory infiltrations on the fallopian tubes are observed in pigs after a recent estrus (Robertson et al. 2006, Katila 2012). The effectiveness of embryos transfer with the original 1.0mm set was confirmed in the study of the development potential of embryos after transplantation and incubation in the uterus (in vivo). A high developmental potential of the blastocysts obtained after 5-day culture in the uterus of transplanted 2- to 4- blastomere embryos has been demonstrated. The same extent of defragmentation of cell nuclei (13-16%) as that found after surgical transplnatation and 5 day in vivo embryo culture was confirmed (Trzcinska et al. 2011).

The apoptotic index of blastocysts following in vivo culture without transfer is comparable (Pomar et al. 2005, Bryla et al. 2010, Bryla and Trzcinska 2012) but, the apoptotic index of blastocysts obtained after in vitro culture is much higher (25% to 90%) than that obtained in our study (Mateusen et al. 2005, Bryla et al. 2009, 2011). The embryo deposition site is determined by the stage of their development. According to a generally accepted procedure, embryos harvested from donors are deposited at the site of their collection or, when embryos are obtained in vitro, the deposition site is determined by the developmental stage of the embryos. In this case, 1- to 8-cell embryos can also be deposited in the oviduct, and morulas or blastocysts in the uterine horn. It has been established that embryos at the later stage of development (morula or blastocyst) can also be deposited into the oviduct (Gajda et al. 2004, 2005). In our study, it was shown that transplantation of blastocysts into the fallopian tube is as effective as depositing blastocysts into the uterus in the initial horn of the uterus (near the mouth of the fallopian tube) and it is similar to depositing blastocysts into the uterus at a length of 1/3 - ½ of uterine corner (Wallenhorst and Holtz 1999). For cryopreserved pig embryos, offspring are produced but the efficiency of the procedure continues to be very low and variable (Hirayama et al. 2020). Higher efficiency was reported when using a special breed of pigs with very high capacity for implantation (Gajda et al. 2005). Our research has shown low effectiveness after laparoscopic vitrified embryo transplantation, which may be more due to the imperfection of the embryo vitrification method of this species (Gajda and Rajska 2014, Whaley et al. 2021) than the shortcomings of the transplant method. The described grasper system enables easy manipulation within the abdominal cavity and best access to the ovaries, oviduct and uterus. Unlike the surgical method, the minimal interference within internal organs results in no adhesions. Experience with pigs, goats and sheep shows that complications following laparoscopic procedures, in the form of adhesions within the peritoneum and internal organs, occur sporadically after 4 to 5 treatments in the same animal (Wieczorek et al. 2018, 2020a). During the transfers of embryos we observed numerous complications. The most common complications, such as bleeding from subcutaneous blood vessels and pneumoperitoneum are easy to remove and do not affect the effectiveness of the methods or the clinical condition of the animals. During transplantation into the uterus, local bleedings were observed. During the bleeding after uterine puncture, hemorrhage outside of the uterine horn was also accompanied by bleeding into the uterine lumen and local inflammation, which could have disrupted the implantation, with possible phagocytosis of the transplanted embryos (Hansen 2011, Faas and de Vos 2017). After the embryos were transferred through the infundibulum, severe damage and massive complications were observed. The walls of the infundibulum are delicate and are damaged even with the use of non-invasive laparoscopic grips. In the available literature, only one study described an effective method for embryo transfer via the infundibulum (33% efficien-

cy) (Besenfelder et al. 1997), but it was not supported by other studies. The method in which embryos are transferred into the oviduct by puncture is much more common (Hazeleger and Kemp 2001, Youngs 2001, Wieczorek et al. 2015).

In conclusion, non-invasive funnel embryo transfer has very limited use due to manual difficulties, damage to the funnel and very low efficiency. During the insertion of the catheter, the fallopian tube is mechanically damaged exhibiting various morphological changes of the fallopian tube, and the severity depends on the size of the catheter. In the case of too thick catheters, the fallopian tube is damaged and dysfunctioned. Laparoscopic methods allow the successful transfer of embryos to the fallopian tube and uterus. The most effective method of laparoscopic embryo transfer is the transfer of early-growing embryos with 2 to 4 blastomeres into the fallopian tube. Laparoscopic transfer of blastocysts into the fallopian tube is as effective as placing them in the uterus.

Acknowledgements

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