

DOI 10.1515/pjvs-2015-0054

Original article

Piglets born after intrauterine laparoscopic embryo transfer

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Abstract

The aim of the study was the preliminary development of laparoscopic transfer of embryos to the uterus in the pig, which can become the alternative for more invasive surgical methods. We proposed the original method of embryo transfer. Donors (n = 40) and recipients (n = 15) of embryos were sows of age of 6-8 months. The estrus cycle of both recipients and donors was routinely synchronized. The experimental animals were divided into two groups. In the first group (10 donors and 3 recipients) embryos were transplanted according to the method described earlier and in the second group (30 donors and 12 recipients) embryos were transplanted according to our own proposed method. As the control group, we used 16 sows after insemination (AI). In animals from both experimental groups pregnancy was diagnosed between 28-31 day after transplantation and in the control group between 28-31 day after insemination. All animals were observed during pregnancy and weaning period in pig farm. Embryos at the development stage of 2-4 cell were obtained surgically and cultured *in vitro* for 4 days. Obtained blastocysts were transferred to donors. The original set of catheters for blastocysts transfer to pig uterus was constructed. Three trocars were placed in abdominal cavity for inserting endoscope and 2 grasps for uterus stabilization. After uterus stabilization, the slide was inserted into abdomen which was used for putting the needle to puncture uterus. Through this needle catheter with embryos was inserted into the uterus cavity. Embryos were placed by injection into lumen of the one uterine horn. From 12 recipients pregnancy was diagnosed in 6 recipients. From 6 litters, 57 piglets were born. We weaned 41 piglets (71.9%). In our study we obtained 50% efficacy, with the mean number of 9.5 alive piglets in litter and 6.8 weaned piglets. The efficacy of developed method of laparoscopic transfer of porcine embryos allows it to be used in routine practice.

Key words: embryo, pig, laparoscopy, transfer

Introduction

The methods of embryos obtaining and transfer in pigs have limited use in routine breeding because of the high fertility of this species and short pregnancy. However, they are widely used in biotechnology of reproduction in this species and they are essential for progress of modern biotechnology. It concerns such fields as: animal breeding, *in vitro* maturation and fertilization of embryos (*IVM/IVF*), oocytes and embryos cryopreservation, animal transgenesis, cloning and experimental medicine. Development and use of these technologies are not possible without simple and effective methods of embryos transfer, supporting practical application of methods mentioned above. On the other hand, the ethic requirements concerning the experiments with animals make it necessary to find and employ the new practical alternative to the old invasive methods and change them to the new ones of the lowest possible invasiveness. Because of these reasons also in pigs it is necessary to improve methods of embryos obtaining and transfer as the alternative for the surgical methods. Surgical methods of embryos transfer have limited application because of their high costs, length of the procedure, significant tissue destruction during surgery, and possibility of postsurgical complications and long period of wound healing. In order to limit surgical interventions as much as possible, low-invasive techniques based on laparoscopy are employed. All over the world, these techniques were being developed for about 25 years (Stein-Stefani and Holtz 1987, Brüssow and Rátky 1996, Besenfelder et al. 1997, Hazeleger and Kemp 2001, Wiczorek et al. 2007).

The aim of the study was the development of laparoscopic transfer of embryos to uterus of pig, which can become the alternative for invasive surgical methods.

Materials and Methods

The study protocol was approved by The II Local Ethic Commission in Krakow 791/2010, September 19, 2010. Donors ($n = 40$) and recipients ($n = 15$) of embryos were sows of age 6- 8 months, body weight 80-110 kg, with no visible symptoms of infection, bred at the same farm. Estrus cycle of both recipients and donors was routinely synchronized and donors were superovulated. The experiment was divided into 4 sessions with 15 animals used per each session, i.e. 10 donors and 5 recipients. During each session 10 donors were used and the numbers of recipients in consecutive sessions were: 3 in the first session, and 4, 3, and 5 recipients during the second, the third and the fourth session, respectively. Experimental animals

were divided into two groups: Group I consisted of pigs operated in session I and Group II consisted of pigs operated in sessions II – IV. As the control group 16 sows after AI were used. Pregnancy was diagnosed in all recipients with USG between 28 and 31 day after embryos transfer. All pregnant recipients were observed during pregnancy and weaning period in pig farm.

The same protocol to prepare an animal to experiment and anesthetic protocol was used in all animals. A feeding was stopped 24 hours before the procedure. A free access to water was assured. Directly prior to the procedure, a canulation of an ear marginal vein was performed (20G, Venflon BD 1.0).

Complex anesthesia was carried out through a premedication with atropine (Atropinum Sulfuricum 0.5 mg, Polfa), dosage 0.06 mg/kg *i.m.* and azaperone (Stresnil, Janssen Animal Health BVBA), dosage 3 mg/kg *i.m.* General infusion anesthesia was used with employment of short acting drugs in all animals after premedication. General anesthesia was induced by natrium thiopental (Thiopental, Biochemie GmbH), dosage 8 mg/kg *i.v.* as a short infusion and subsequent 1/4 to 1/5 of initial dosage, depending on the strength of drug effect. A constant control of respiratory and circulatory systems was performed because of depressive impact of thiopental on respiration and circulation.

Embryos of two to four cell stage were obtained surgically by flushing out from the Fallopian tube. The laparotomy was performed in *linea alba*, the ovaries with oviducts were taken out of the abdominal cavity. In each ovary, the numbers of ovulations were recorded. The oviducts were flushed with 20 ml of PBS with 4% bovine albumin (Albumin from bovine serum, Sigma). The flushed oviducts were put back to the abdomen, the wound was closed with absorbable sutures (PGA2, Yavo). Embryos were collected into collective dishes, counted, evaluated under stereoscopic microscope and transferred to a culture. The quality of all obtained embryos was estimated under stereomicroscope (magnification 50X). Not-fertilized oocytes or degenerated embryos were rejected. All qualified embryos were cultured *in vitro* for 4 days, all under the same conditions, in a NCSU-23 medium (Petter and Wells, 1993) suitable for pig embryos culture in incubators at 39°C, in the atmosphere of 5% CO₂ in air. Blastocysts obtained during culture were evaluated and transferred to donors.

Transfer of embryos with endoscopy

The method described before (Besenfelder et al. 1997) was used during the session I of the study. Because we had no pregnancies with the method

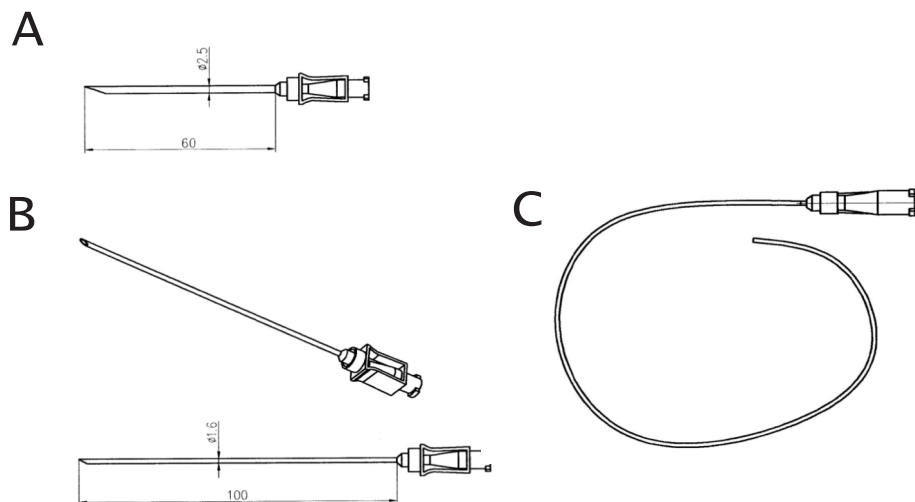


Fig. 1. The three-piece set for laparoscopic embryo transplantation: A – the slide for puncture of abdominal wall, B – needle for puncture the uterine horn, C – elastic catheter for placed embryo in lumen of the uterine horn.

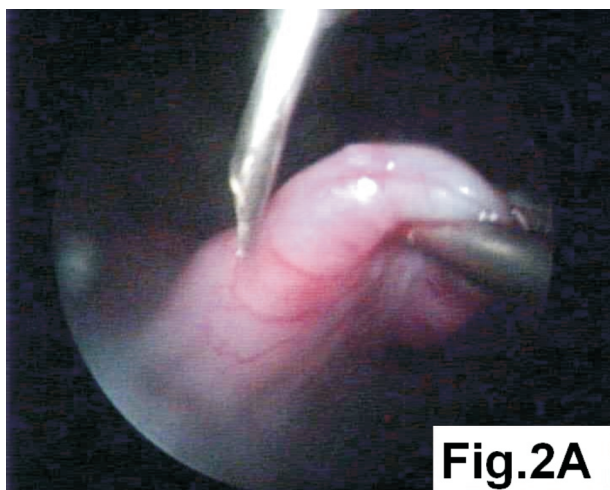


Fig.2A

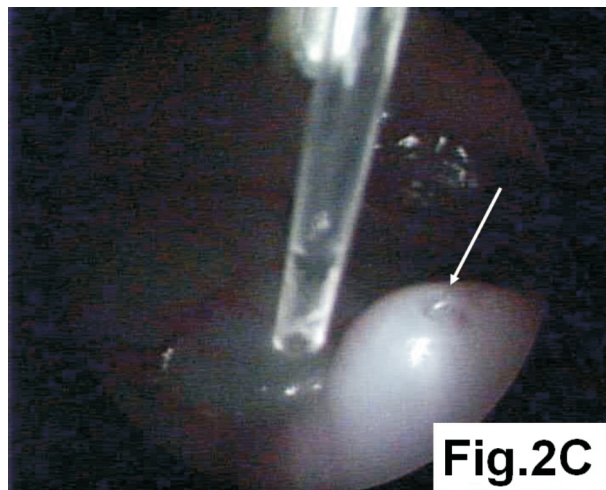


Fig.2C

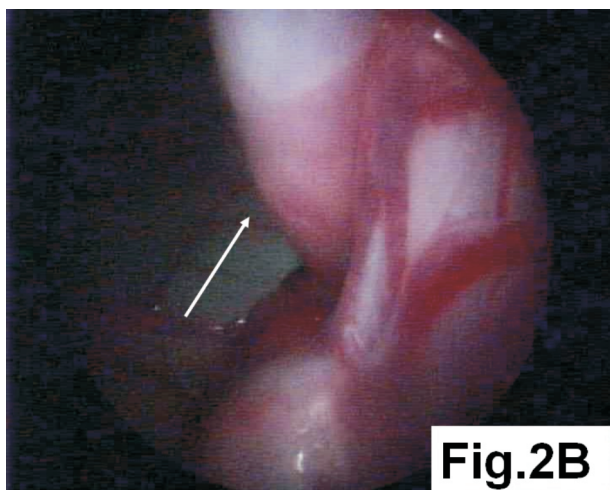


Fig.2B

Fig. 2. The intrauterine laparoscopically transfer embryo in pig: A – a horn of uterus before an insertion of a catheter, B – a catheter inside lumen of uterus (the end of catheter visualized by arrow), C – the elastic catheter after removal from uterus (A place after insertion the catheter visualized by arrow)

described, we proposed the new method of embryos transfer. The new original three-piece set of catheters (Fig. 1) with original method of embryos transfer were developed. Three trocars were inserted into abdominal cavity. They were used for inserting endoscope and 2 grasps for uterus stabilization. An endoscope was inserted as the first, between the 3rd and 4th pairs of nipples, 5-10 cm to the left from the middle line of abdomen. After inserting endoscope, abdominal cavity was filled with gas (filtered air) and evaluation

Table 1. Results of intrauterine laparoscopic method of pig blastocysts transfer.

Group	Recipients number	Number of embryos transferred to 1 recipient	Number of pregnant recipients (%)	Born piglets	Number of born piglets /1 recipient	Alive piglets	Alive piglet/1 recipient	Deaths of piglet after delivery	Number of dead piglet/1 recipient	Weaned piglets	Weaned piglet/1 recipient
I (Session I)	3	36	0 (0.0%) ^{A,B}	-	-	-	-	-	-	-	-
II (Session II-IV)	12	32.7	6 (50%) ^{A,C}	57	9.5 ^C	47 (82.5%) ^C	7.8 ^C	6 (10.5%)	0.85	41 (71.9%)	6.8 ^C
Control* (Inseminated pig)	16		15 (93%) ^{B,C}	187	12.5 ^C	171 (91.4%)	11.4 ^C	13 (7.0%)	0.87	158 (84.75%)	10.53 ^C

Control – data from 15 farrows born after AI (Insemination).

Statistical data: A – a statistical significance between I group and II group ($p \leq 0.05$), B – a statistical significance between I group and Control group ($p \leq 0.05$), C – a statistical significance between II group and Control group ($p \leq 0.05$).

of internal organs was performed. After this evaluation, 2 additional trocars were inserted, one in the middle line of abdomen between the 2nd and 3rd pairs of nipples, the second one 5-10 cm to the right from the middle line of abdomen, between the 4th and 5th pairs of nipples. Grasps for stabilization of uterine horn were inserted through these trocars. Uterine horn was stabilized by clamping the wide ligament of uterus, at the edge of the corner, at the first 1/4 – 1/3 of its initial length. After uterus stabilization, the slide (Fig. 2A) was inserted into abdomen which was used for inserting the needle to puncture the uterus (Fig. 2B). The uterine horn was punctured and the elastic catheter (Fig. 2C) with embryos was inserted through this needle into lumen of uterine horn to a depth of 3-5 cm. Embryos were placed at the beginning of catheter (1-2 cm) in the minimal amount of medium. They were placed by injection into the uterus cavity. Embryos were transferred into one uterine horn. At one time, an average number of 36.4 blastocysts was placed in a lumen of the uterine horn. After embryos insertion, catheter, grasps and trocars were removed in inverse sequence to their insertion. After trocars removal, simple single sutures were made. Peritoneum and muscles were not sutured as the wound size was very small (5-10 mm). Pregnancy was diagnosed with USG between 28 and 31 day after embryos' transfer.

Statistics

The statistical analysis was performed using PS Imago software. The-Student test was used to compare the differences between the tested groups (confidence interval 95%).

Results

The results obtained are presented in Table 1 and Fig. 3.



Fig. 3. The first piglets in Poland born after laparoscopic transfer embryo.

We found 744 ovulations and 577 embryos (77.5%) obtained from 40 donors after synchronization of oestrus cycle and superovulation. After initial evaluation, 575 embryos were qualified for *in vitro* culture. Two embryos were rejected because of cells degeneration. After 4 days of *in vitro* culture, 547 embryos were at the stage of blastocyst. Mean number of 36.4 embryos was transferred per one recipient. Embryos were transferred with laparoscopic method to 15 recipients, 3 of them were transferred by method described earlier (Besenfelder et al. 1997) (session I/Group I), remaining 12 by the new method (session II – IV/Group II). None of the first 3 recipients was pregnant. In the group of the next 12 recipients a pregnancy was diagnosed in 6 recipients (50%) ($p \leq 0.05$ for each comparison: I/II groups, I/Control

groups and II/Control Groups;). From 6 litters in II group 57 piglets were born, 47 alive and 10 dead. (9.5 piglets per one recipient; 7.8 alive born piglet per one recipients; ($p \leq 0.05$ in comparison to control group). In Group II, among all piglets that were born alive, 3 piglets died during the first 3 days after delivery, 1 because of acute diarrhea and 2 crushed by their mothers and next 3 piglets died between the third and the fourth weeks of life because acute diarrhea. We weaned 41 piglets (71.9%; the statistical significance vs control group ($p \leq 0.05$) (Fig. 3).

Discussion

The laparoscopic method of pig blastocyst transfer to uterus was used for the first time in Poland in the presented study. The method of blastocysts transfer to pig uterus and set of catheters have been submitted to The Patent Office of the Republic of Poland and registered as P392128.

The majority of used techniques is based on employment of modified catheter for intrauterine insemination or modified intravenous catheter or original sets which are used for embryos introduction after inserting them to uterus (Sims and First 1987, Ratky and Brussow 1995, Besenfelder et al. 1997, Ratky et al. 2001, Riha and Vejnar 2003). Preliminary it was planned to use the method described before (Besenfelder et al. 1997). In this method, three trocars were used. Places of their insertion had a shape of a triangle with sides 8-10 cm long. Two trocars were placed in the abdominal middle line and the third one was placed laterally. Foremost trocar was used for an endoscope insertion, ventral and lateral ones – as tools stabilizing ovaries and uterus horn as well as catheters for embryos' transfer. The effectiveness of this method was 33-42%, measured as a number of obtained pregnancies, with mean number of piglets in one litter of 6.1-7.6 (Besenfelder et al. 1997). In our study none of the three recipients of transferred embryos was pregnant after embryos transfer performed according to the described method (Table 1). Inserting of endoscope camera in one line together with one of grasps caused difficulties in manipulation within internal organs and did not allow a free access to uterus horns, fallopian tubes and ovaries. Anatomical barriers are also the additional difficulty in laparoscopy in the area of pig uterus. Long horns of uterus (about 50-100 cm) are the reason why low-invasive methods in pigs are extremely difficult and require high experience of surgeons. For this reason we proposed our own original method. The approach described above allowed to get a free access and manipulations in abdominal cavity. The original

three-part set of catheters for embryos introduction was developed for our method. Uterus wall was punctured with needles that were used for inserting the elastic catheter with embryos. Catheter was inserted 3-4 cm deep. The construction of this set gives a possibility to put a catheter with embryos to an uterus cavity directly after puncture of the wall of uterine horn and limits a contact of embryos with external environment to a few seconds needed for removing them from medium and placing into uterus lumen. An appropriate elasticity of catheters is the requirement to perform a safe procedure of embryos transfer because it allows putting the catheter deep enough and placing embryos without risk of perforation or mechanical destruction of uterus corner. Rigid catheters cannot be inserted deep enough because of the risk of damage of uterus horns. Moreover, too shallow deposition of embryos can effect in outflow of embryos outside the uterus during catheter removal. The optimal depth of embryos' deposition is 3-5 cm. In our study, embryos were placed at the proximal part of uterine horn in 1/3 of length of uterine horn from an oviduct isthmus. Too deep deposition of embryos can be unfavorable and cause lower number of pregnancies and number of piglets in one litter (Stein-Stefani and Holtz 1987, Wallenhorst and Holtz 1999). This is the reason for statement that the optimal place of pig embryos deposition is the proximal or the medium part of uterine horn (Stein-Stefani and Holtz 1987, Wallenhorst and Holtz 1999). In our study embryos were transferred into one uterine horn because migration of embryos from one uterine horn to the other is known to occur in swine (Dziuk et al. 1964). In the case of transfer of embryos to uterus there is the possibility of deposition of embryos in more advanced stage of development, i.e. late morula or early blastocyst. It is assumed that embryos should be put to the place of their obtaining. If they were obtained *in vitro*, their age and development stage is crucial for choosing place of deposition. In this case, the most suitable place of deposition of embryos containing 1-8 cells is Fallopian tube and for more mature embryos the uterus horn. As the result, our method makes it possible to deposit more mature embryos i.e. late morula or early blastocysts into uterus.

The transfer of embryos at the stage of morula or blastocyst, in comparison to embryos containing 4-8 cells, led to improvement of method efficacy (Hazeleger and Kemp 1994, Li et al. 1996). In case of sows, deposition of blastocysts of the higher diameter leads to the higher ratio of pregnancies in comparison to deposition of embryos of the smaller diameter (Hazeleger et al. 1999). In most methods, the number of transferred embryos is relatively high, up to 40 (Dziuk et al. 1964, Hazeleger and Kemp 1994, 2001,

Rátky and Brussow 1995, Besenfelder et al. 1997, 2010). Embryos are transferred in small volume of medium 0.1-0.3 ml. Such small volume is also sufficient for transfer of the high number of embryos (Hazeleger and Kemp 2001, Youngs 2001).

In our study we obtained 50% efficacy, with the mean number of 9.5 live piglets in litter and 6.8 weaned piglets. Our results are similar to these obtained by other authors. The mean efficacy of laparoscopic transfer of pig embryos to uterus is 35-41%, with the mean number of 6.5 to 7.5 of alive piglets in litter (Sims and First 1987, Besenfelder et al. 1997, Riha and Vejnar 2003). Although the efficacy of our method is similar to results reported by other authors, it is still lower than efficacy of routinely used surgical method, with its mean efficacy of 50-60% (Huang et al. 2002). It is caused by the fact that pigs, among all animal breeding species, are the one in which laparoscopy is the most difficult to perform. The main difficulties are anatomical barriers. The exceptional length of uterus corners with relatively low volume of abdominal cavity are the main causes why low invasive methods in pigs, with efficacy similar to surgical methods, are technically very difficult and require high manual skills of surgeons performing this procedure.

The proposed method of embryos' transfer was developed as the alternative to the standard surgical method. Up till now, the routine method of pig embryos transfer was the surgical method, in which access to uterus is obtained after performing 5-10 cm incision in *linea alba* and taking uterus out of the abdomen. This method has limited application because it is connected with high tissue trauma, long period of wound healing and very high risk of postsurgical complication, mainly massive adhesions in uterus and Fallopian tubes, but also hernias, local abscessus and scars at the place of laparotomy (Baril et al. 1989, Tervit 1996, Besenfelder et al. 1997, Huang et al. 2002). Implementation of laparoscopic method allowed for elimination of limits and complications of the surgical method. The main advantage of the proposed method is limitation of surgical intervention to the necessary minimum. In procedure described by us, wounds of muscles of abdomen and peritoneum after trocars removal, with diameter of 5-10 mm, do not require sutures and can be left for self-healing. Single, simple sutures are applied for the skin. The atraumatic method of uterus corners stabilization is also secured. As the effect, adhesions and other complications of surgery are eliminated. During 30 days of observation, in 15 recipients we did not observed formation of adhesions and scars after performed procedure. The atraumatic stabilization of uterus horn is assured, too. According to literature, laparoscopic

method allows for shortening of time of the procedure (Nellenschulte and Neimann 1992, McMillan and Hall 1994, Besenfelder et al. 1997, 2010), it can be performed in form environment (Baldassarre 1999), it allows for multiple procedures in the same donors and recipients (Baril et al. 1989, Wiczorek et al. 2010), and it is safe for animals because of the minimal dosage and short time of infusion of anesthetic drugs (Rátky et al. 2001). It decreases the risk of infectious disease transmission (Bielanski 2012) and costs connected with embryos transfer as well as it lets to obtain genetic material of good quality (Baldassarre et al. 1999, Bari et al. 2000).

In our study, the laparoscopic transfer of embryos resulted in the birth of live piglets. Implementation of this method lets to eliminate disadvantages of surgical methods connected with high tissue trauma and postsurgical complications. Although the efficacy of the method, after preliminary analysis, allows obtaining relatively high percentage of pregnancies, it is still lower than for surgical methods. However, further development of this method and employed modification should allow reaching efficacy of 50-60% which is comparable to surgical methods.

Acknowledgments

This work was financially supported by the Polish Ministry of Science and Higher Education, project No. N N302 623140.

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