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4

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6

7

Reproductive ability of minipigs as surrogates for somatic cell nuclear transfer

Running Title: Features of minipigs as surrogates

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Abstract

Pigs are genetically, anatomically, and physiologically similar to humans. Recently, pigs are in the spotlight as a suitable source animal for xenotransplantation. However, to use pigs as source animals, pigs should be raised in designated pathogen-free facilities. There is abundant data from embryo transfer (ET) experiments using farm pigs as surrogates, but data on ET experiments using minipigs are scarce. Eighty minipigs were used for ET experiments and after transplantation, the implantation and delivery rates were investigated. It was also confirmed whether the pregnancy rate could be increased by changing the condition or surgical method of the surrogate. In the case of minipigs that gave birth, the size of the fetal sac on the 28th day of ET was also measured. The factors that can affect the pregnancy rate such as estrus synchronization program, ovulation status at the time of ET, the number of repeated ET surgeries, and the ET sites, were changed, and the differences on the pregnancy rate were observed. However there were no significant differences in pregnancy rate in minipigs. The diameter of the implanted fetal sac on the 28th day after ET in the minipigs whose delivery was confirmed was calculated to be 4.7 ± 0.5 cm. In conclusion, there were no significant differences in pregnancy rate of minipigs in the comparative experiment on various factors affecting the pregnancy rate. However, additional experiments and analyses are needed due to the large individual differences of the minipigs.

Keywords: embryo transfer, fetal sac diameter, minipig, pregnancy

Introduction

The pig (*Sus scrofa*) is an omnivorous, monogastric mammal [1] that is anatomically, biochemically, physiologically, and pathologically similar to human [2, 3]. There are about 300 breeds worldwide, and their weight varies from 50 kg to 350 kg, depending on the breed. Based on their sizes, they are categorized into the large breed, medium breed, and small breed. Currently, most of the pigs raised on the farms are Landrace, Yorkshire, Duroc, and their hybrids. These hybrids are easy to breed, give rise to a lot of livestock, and are economical because they grow faster than other breeds [4].

In general, in a transgenic pig generation using somatic cell nuclear transfer (SCNT), a farm pig is used as a surrogate mother due to the high accuracy of the estrous synchronization program that is established over a long period, the ability to conceive as a surrogate mother have been proven. In the transgenic pigs, as the source animal for xenotransplantation, controlling the zoonotic pathogens are important. In this respect, the control of pathogens derived from the surrogate mother is difficult when farm pigs are used as surrogate mothers. [5]. To prevent infection, at least the sows should be raised in specific pathogen-free (SPF) facilities and used as surrogate mothers and the presence of infectious agents should be monitored through periodic pathogen screening of the sows. However, in the case of using sows from farms, it is not economical in terms of scale and operation of the SPF facilities due to the size of the individual sow. On the other hand, minipigs have a great advantage in that they are relatively small, weighing 32-140 kg compared to farm pigs [6, 7], and are easy to breed in SPF or designated pathogen-free (DPF) facilities as experimental animals [1, 8]. In the case of minipigs, they are already being used in research in various medical-related fields such as toxicology, pharmacology, experimental surgery, and xenotransplantation [9]. However, compared to standardized farm pigs, minipigs may have large individual differences

in their sizes, fewer offspring, and low reproductive efficiency, since minipigs are not developed for breeding [8, 10].

In pigs, most estrus occurs spontaneously, and once estrus begins, it repeats in a cycle of 18 to 21 days. When estrus synchronization becomes possible, various artificial reproductive technologies (ARTs) using frozen semen, sex-differentiated semen, or transgenic embryos can be applied to pigs [11]. Therefore, over many years, highly purified human chorionic gonadotropin (hCG), partially purified pituitary isolates (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)), synthetic gonadotropin-releasing hormone (GnRH) and GnRH analogs have been used to induce estrus. In pigs, estrus synchronization has been applied in various ways depending on the maturity and the degree of follicle development in the individual [12]. Estrus is a highly regulated process that occurs due to the interaction of various hormones. Briefly, GnRH is secreted from the hypothalamus, and followed by FSH and LH secretion from the anterior pituitary gland due to stimulation by GnRH. The size of the ovarian follicle grows and matures by the secreted FSH and LH, leading to ovulation. Estrogen is secreted from the growing follicle, and various signs of estrus (e.g, redness and swelling of the vulva, standing or immobilization response, LH surge, and ovulation) are exhibited by this hormone. After ovulation, pregnancy is maintained by progesterone secreted from the corpus luteum, or when pregnancy is not achieved, the corpus luteum rapidly degenerates and enters the next estrus phase by Prostaglandin F2α (PGF2α). Predicting and controlling these hormonal changes is a cumbersome task, but synchronization of estrus is essential for the transfer of scheduled-produced transgenic pig embryos into the surrogates on heat within the due date [13-16].

SCNT is a technology that removes the nucleus and polar body from an *in vitro* matured oocyte to produce an oocyte that is lacking genetic material, then involves inserting a somatic cell and fusing them to create a newly fertilized embryo [17, 18]. Transgenic animals can be

produced by inserting transgenic somatic cells. Using this method, 1) genomic research through gene expression model production, 2) therapeutic drug development through disease model animal production, and 3) source animal production for xenotransplantation through immune-modulated transgenic animal production can be performed. Recently, with the discovery of CRISPR/Cas9, the production of transgenic animals through SCNT has been accelerated, and transgenic animals are being made from various animals (e.g, sheep [19], cow [20], mouse [21], goat [22], pig [23], dog [24], camels [25], and monkey [26], etc.). However, even when farm pigs with high fertility efficiency are used as surrogates, the production rate of transgenic animals through SCNT is too low. In addition, it was reported that the fusion rate decreased when the SCNT procedure was performed on minipig somatic cells on farm pig oocytes [27]. This means that the probability could be lower if minipigs were used as surrogates.

Therefore, this study intends to compare the different factors such as implantation and delivery rate when minipigs are used as surrogates for the production of transgenic pigs. In addition, we try to find out what factors affect the implantation and delivery rate in minipigs, and to find a way to increase the overall pregnancy rate.

Materials and methods

Ethics statement

The experimental protocols were approved by the International Animal Care and Use Committee of Apures Inc (APURES-IACUC 200709-001, 210506-001, and 220420-001). Minipigs were used as surrogate mothers raised in Apures' SPF facility (Pyeongtaek, Korea).

Estrus synchronization program

The selected surrogate mothers were fed for 18 days by adding Altrenogest (MSD, Seoul, Korea) to the feed at the rate of 5 mL/head (once in the morning) per day. For subjects

who received Altrenogest for 18 days, 5 mL/head of PG-600 was intramuscularly injected to induce estrus after a rest period of 1 day. After injection, a visual check for the estrus was performed for 4 to 5 days, and selected a surrogate mother for surgery (Figure 1).

Somatic cell nuclear transfer (SCNT)

To produce cloned porcine embryos, donor cells were subjected to SCNT, which was done following the protocol previously established in our studies with a slight modification [28]. Briefly, immature oocytes were obtained from pig ovaries from slaughterhouse and cultured for 40 hrs to induce maturation. The *in vitro* matured oocytes were enucleated using an aspiration pipette, then microinjected with transfected donor cell, fused by electrical stimulation, and further activated using an electrical protocol. The resulting activated embryos were cultured for 7 days. The embryos were evaluated for cleavage on Day 2 and blastocyst formation on Day 7, and the total cell number of cloned blastocysts were counted on Day 7.

Embryo transfer (ET)

The surrogate minipig was restrained, and anesthesia was induced by injecting ketamine (5 mg/kg; Yuhan, Seoul, Korea) and xylazine (1 mg/kg; Cat. No. 86140632-01, Bayer, NJ, USA) into an ear vein, as previously described [29]. After intravenous injection, the unconscious pig was placed on a surgery table in a ventrodorsal posture. General anesthesia was maintained with isoflurane (Hana Pharm, Seoul, Korea) under the supervision of a veterinarian. Up to 300 reconstructed embryos were loaded into a Tomcat catheter (Cat. No. sc-363807, Santa Cruz Animal Health, TX, USA) with PZM-3 equilibrated in 5% CO_2 with an air cushion. The embryos were placed into the uterine tubes of each surrogate animal through a Tomcat catheter via a small puncture made with a suture needle (Cat. No. 6307-71; Covidien, MA, USA).

Progesterone analysis

Blood samples were collected at the time of ET surgery. While under general anesthesia, blood samples were collected from the jugular veins of surrogate pigs using 18-gauge needles connected to disposable syringes. The samples were put into serum-separating tubes (Cat. No. 367955, BD Biosciences, NJ, USA), centrifuged 5,000 × g for 10 min at 25°C to separate serum from blood after clotting, and were delivered to the laboratory at 0°C in an ice box. The samples were then transported to an analysis center (Neodin Medical Institute, Seoul, Korea) to measure the P4 concentration.

Statistical analysis

All results are presented as the mean ± standard error (SE). Statistical significance was estimated using the chi-square test, unpaired t-test, and analysis of variance. All statistical analyses were performed using GraphPad Prism 8 (ver. 8.3.0; GraphPad Software, CA, USA) and p-values of <0.05 were considered to be statistically significant.

Results

Estrus synchronization program differences and pregnancy rates in minipigs

Since transplantation is performed through surgery, ovulation was accurately confirmed by visually observing the condition of the ovaries. Even though the estrus synchronization program was used, minipigs in pre-ovulation were 80.4% (37/46), in mid-ovulation were 15.2% (7/46), and in post-ovulation were 4.3% (2/46). Most of them were confirmed to be in the pre-ovulation state. Therefore, the estrus synchronization program was conducted one day earlier, and the difference in the pregnancy rates was investigated. In

addition, we observed the changes in progesterone concentration according to the change of the estrus synchronization program.

Of the 80 experimental groups, a total of 57.5% (46/80) minipigs were induced for estrus synchronization one day (i.e, on 'Day 0') and 42.5% (34/80) minipigs one day earlier (i.e, 'Day -1'). Implantation rates were observed between 'Day 0' and 'Day -1' with 32.6% (15/46) and 58.8% (20/34), respectively. Delivery rates were found between 'Day 0' and 'Day -1' to be at 20.0% (3/15) and 15.0% (3/20), respectively. (Table 1. Estrus synchronization program section). In all the factors which were compared, there are no statistically significant differences. The difference in the concentration of progesterone according to the changes in the estrus synchronization program was 1.984 ± 0.694 ng/mL in the 'Day 0' group (n=12) and 4.283 ± 1.380 in the 'Day -1' group (n = 20) with no statistically significant difference between groups (Figure 2a and Supplementary Table 1).

Progesterone concentrations in minipigs were 4.185 ± 1.571 ng/mL, 2.555 ± 0.799 ng/mL, 1.908 ± 0.811 ng/mL, and 3.704 ± 1.801 ng/mL when implantation failed, implantation, miscarriage, and delivery, respectively with no significant difference (Figure 2b and Supplementary Table 2).

Factors that affect pregnancy in miniature pigs

In order to increase the production efficiency of transgenic pigs from minipigs, the correlation of various factors with pregnancy-related factors was compared. First, the state of ovulation in the minipig ovary at the time of ET surgery was observed and the relationship between ovulation status and pregnancy was confirmed. In a total of 80 minipigs, 85.0% (68/80) pigs were identified to be in pre-ovulation, 11.3% (9/80) pigs in mid-ovulation, and 3.8% (3/80) pigs in post-ovulation. Implantation rates were confirmed in 45.6% (31/68), 33.3% (3/9), and 33.3% (1/3) pigs with pre-, mid-, and post-ovulation, respectively. Delivery rates were 16.1%

(5/31), 0.0% (0/3), and 100.0% (1/1) pigs with pre-, mid-, and post-ovulation, respectively. However, in the all factors which were compared, there are no statistically significant differences. In minipigs, there was no correlation among the implantation and delivery rates according to the ovulation status of the ovaries (Table 1. Ovulation status section).

Next, the relationship between the number of ETs and pregnancy was examined. Of a total of 80 minipig surrogates, 46.3% (37/80) were the first to undergo ET, 37.5% (30/80) to the second ET, and 16.3% (13/80) to the third ET. Implantation rates were confirmed in 37.8% (14/37), 43.4% (13/30), and 61.5% (8/13) minipigs after the first-, the second-, and the third-operation, respectively. The rate of delivery was confirmed as 7.1% (1/14), 30.8% (4/13), and 12.5% (1/8) minipigs after the first operation, the second operation, and the third operation, respectively. However, among the factors which were compared, there are no statistically significant differences. In minipigs, there was no correlation among the implantation and delivery rate rates according to the number of ET (Table 1. Number of surgeries section).

Finally, it was checked whether implantation of SCNT- embryos into one fallopian tube and transplantation into both fallopian tubes could affect pregnancy. When transplanted into a single fallopian tube, approximately 300 transgenic embryos were implanted in one fallopian tube, and when transplanted into both fallopian tubes, 150 embryos were transplanted into the right fallopian tube, and the remaining 150 embryos were transplanted into the left fallopian tube. We unified the number of embryos implanted in one surrogate mother to about 200. A total of 80 surrogate mothers were identified, 85.0% (68/80) were transplanted embryos into one fallopian tube, and 15.0% (12/80) were fertilized embryos in both fallopian tubes. The implantation rates were 36.8% (25/68) and 83.3% (10/12) when transplanted on a single side and transplanted on both sides, respectively. The delivery rates were 16.0% (4/25) and 20.0% (2/10) when transplanted on a single side and transplanted on both sides, respectively. (Table

1. Embryo transfer sites section). There were no significant differences in implantation and delivery rate by embryo transfer sites.

Fetal sac diameter of the miniature pigs at 4 weeks after embryo transfer

So far, the fetal sac diameter of transgenic fertilized embryos around day 28 in minipigs has not been reported. In this study, 28-day fetal sac diameters were measured using a retrospective method from a total of 6 minipigs that had completed delivery using transgenic fertilized embryos, and a result of 4.7 ± 0.5 cm was obtained (Total number of fetal sacs checked n = 10) (Figure 3 and Table 2).

Discussion

In the case of implantation rate, according to the estrus synchronization program, 'Day 0 : Day -1 = 32.6% (15/46) : 58.8% (20/34)', and according to embryo transfer sites, 'Single oviduct : Both oviduct = 36.8% (25/68) : 83.3% (10/12)' were observed, respectively. One of the two groups may have numerically higher results, but no statistically significant differences were found. These are probably due to the small number of minipigs used in the experiment. It was also confirmed if the estrus synchronization program was started a day earlier, the concentration of progesterone at the time of ET was increased. However, it would be better to start the estrus synchronization program a day earlier, considering that implantation rates and delivery rates are not significantly different from those of the group that did not start the estrus synchronization program a day earlier.

In minipigs, it was confirmed that there was no significant correlation between the concentration of progesterone at the time of ET, the estrus synchronization program, and the ovulation state of the ovaries. This may be due to the small number of individuals in the minipigs whose progesterone concentration was measured, but it was confirmed that there was

241 a large difference between the individuals responding to the estrus synchronization program as
242 the range of progesterone concentration between the individuals was large. This means that
243 since the minipigs used for ET have not yet been inbred, there are differences in genetic,
244 physiological, and reproductive characteristics of each individual.

245 It was confirmed that there was no significant difference in pregnancy statuses
246 regardless of the ovulation status of the ovaries confirmed at the time of ET in minipigs, the
247 number of surgeries performed, and whether the ET was performed using one fallopian tube or
248 both fallopian tubes. In the case of miscarriage rates among pregnancy statuses, the influence
249 of other factors, such as the transgenic technology used in the establishment of the donor cells
250 for SCNT and the type of transgene, may be greater than the effect of the surrogate itself. As
251 mentioned above, in the case of minipigs, it is thought that there are many differences in
252 individual characteristics because they have just been used as experimental animals. As data
253 from more individuals are accumulated, there is a possibility that significant differences can be
254 identified in the experiments, and it is a future task to find an optimal method for using minipigs
255 as a surrogate based on these data.

256 According to Knox *et al*, the fetal sac diameter in pigs gradually increased from the
257 18th day to the 29th day of gestation, reached a peak at 6.5 cm, decreased until the 39th day,
258 and started to increase again from the 42nd day [30]. In order for transgenic fertilized embryos
259 to develop properly to the end, it was confirmed that the fetal sac diameter should be about 4.7
260 ± 0.5 cm on the 28th day of diagnosis after implantation (ET), and the appearance of the fetus
261 was observed in many cases (Figure 3). This is a smaller size than 6.5 cm in farm pigs, but it
262 is thought to be smaller in fetus size due to the difference according to subspecies. It was
263 observed that implantation can be diagnosed if the fetal sac diameter is greater than 1.0 cm by
264 day 28, but this small fetal sac does not lead to delivery in many cases. Finally, to bring
265 transgenic pigs into the DPF facility, live offspring production using SCNT and C-sec will be

performed, and to control the source of infection, it was determined to use pigs raised in at least SPF facilities as surrogates. Therefore, minipigs currently managed in SPF facilities were used for the experiment. However, it was confirmed that the minipigs had lower fertility and delivery rates compared to farm pigs which are specialized for breeding. To resolve this, various factors that can affect fertility in minipigs have been tested, but no definitive solution has been found so far. One of the main reasons for this is that the ET method developed mainly for farm pigs so far is not applied equally to minipigs with different genetic or reproductive physiology. In addition, the difference between donor cells used in SCNT, transgenic technology used in the establishment of donor cells [31], and the number of the target genes in donor cells may have affected the pregnancy rate. Further research is needed on how to increase the pregnancy efficiency of minipigs while continuously evaluating various factors.

In conclusion, it was confirmed that there was no effect on implantation and delivery rates when the estrus synchronization program, ovulation status, number of surgeries, and embryo implantation site were changed in minipigs. Furthermore, it was confirmed that there was no correlation between the pregnancy statuses and the concentration of progesterone at ET surgery. In order for a transgenic fertilized embryo to develop into full term in minipigs, it should be about 4.7 ± 0.5 cm on the 28th day, and a fetus is mostly observed in the fetal sac.

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Conflict of interest

The authors declare no conflicts of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Formal analysis: JM, S-JK, BC

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Methodology: JM, S-JK

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Writing - review & editing: JM, S-JK, JL, HYK, BC, SJK

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Figures

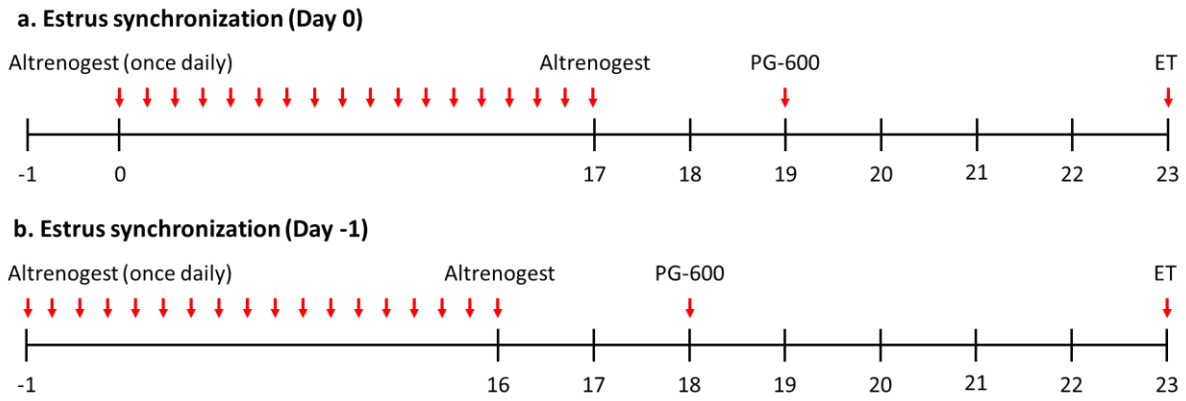

Figure 1. Illustration of estrus synchronization program.

(a) Estrus synchronization program started on 'Day 0'

From Day 0 to Day 17 (total of 18 days), Altrenogest is mixed with feed and fed once a day, then no treatment on Day 18, and muscularly injected with PG-600 on Day 19. After that, estrus is visually checked for about 4 days, and transplantation is conducted on Day 23.

(b) Estrus synchronization program started on 'Day -1'

From Day -1 to Day 16 (total of 18 days), Altrenogest is mixed with feed and fed once a day, then no treatment on Day 17, and muscularly injected with PG-600 on Day 18. After that, estrus is visually checked for about 5 days, and transplantation is conducted on Day 23.

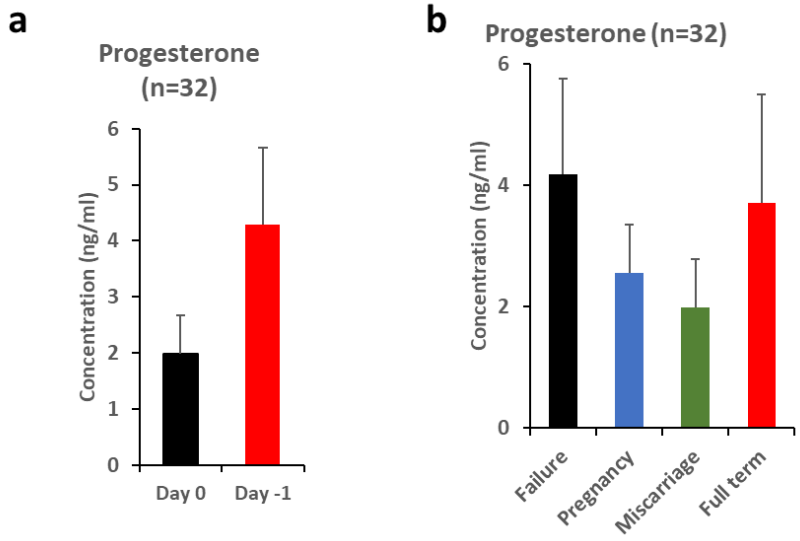

Figure 2. Progesterone concentration differences between estrus synchronization program and pregnancy statuses.

(a) Progesterone concentration related to estrus synchronization program

(b) Progesterone concentration differences among pregnancy statuses

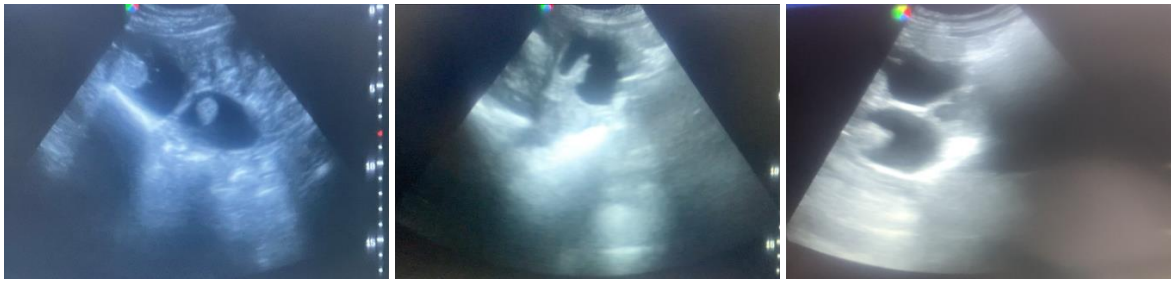

Figure 3. Three representative images of fetal sacs on the 28th day after ET of the miniature pig. When the average of the longest diameters was obtained from a total of 10 fetal sacs, it was measured to be 4.7 ± 0.5 cm.

Tables

Table 1. Differences in pregnancy and delivery rates according to multiple factors

Factors		No. of surrogates	No. of pregnancy (Implantation rate)	No. of Delivery (Delivery rate)
Estrus synchronization program	Day 0	46	15 (32.6 %)	3 (20.0%)
	Day -1	34	20 (58.8%)	3 (15.0%)
	Total	80	35 (43.8%)	6 (17.1)
Ovulation status	Pre-ovulation	68	31 (45.6 %)	5 (16.1%)
	Mid-ovulation	9	3 (33.3%)	0 (0.0%)
	Post-ovulation	3	1 (33.3%)	1 (100.0%)
	Total	80	35 (43.8%)	6 (17.1%)
Number of surgeries	First	37	14 (37.8 %)	1 (7.1%)
	Second	30	13 (43.4%)	4 (30.8%)
	Third	13	8 (61.5%)	1 (12.5%)
	Total	80	35 (43.8%)	6 (17.1%)
Embryo transfer sites	Single oviduct	68	25 (36.8%)	4 (16.0%)
	Both oviduct	12	10 (83.3%)	2 (20.0%)
	Total	80	35 (43.8%)	6 (17.1%)

Table 2. Fetal sac diameter on 28th day after embryo transfer

No.	Fetal sac length (cm)
1	6.2
2	5.2
3	6.5
4	4.8
5	4.8
6	6.0
7	5.2
8	2.0
9	3.0
10	2.8
Mean ± SE	4.7 ± 0.5