Effects of FSH Treatment on Egg Retrieval and Quality, in vitro Fertilization, and Ovulation of SMB Synchronized Ewes

T.K. STENBAK, A.S. ERICKSON, H. BERGINSKI, M.J. TOUTGES, D.A. REDMER, L.P. REYNOLDS, and A.T. GRAZUL-BILSKA

Dept. of Animal & Range Sciences, North Dakota State University, Fargo, N D 58105

INTRODUCTION

The world population is increasing at dramatic rates; therefore the demand for food will continue to grow. In the United States, not only is the population increasing, but so is the average annual income. Consumers have more money to spend; they do not want to buy more food but instead want more specialized products. The consumers are demanding a more uniform, higher quality, nutritious product at a low price, therefore, requiring producers to raise animals that meet these specific demands. Altering herd genetics is a slow and expensive process because of the limitation of having a set number of offspring produced by each animal. However, through the use of assisted reproductive technology (ART) each animal has the ability to produce an increased number of offspring, thereby allowing the producer to more easily alter herd genetics to meet the consumers demands. Using ART, livestock producers will be able to increase the number of offspring produced by two genetically superior parents.

In recent years, there have been new introductions and improvements to ART for the creation, advancement and preservation of animal genetics as well as for the continued improvement of animal reproductive efficiency (Gordon, 1997). In sheep reproduction, ART has been introduced to obtain multiple embryos, in vivo or in vitro, and to obtain transgenic or cloned animals (Schnieke et al., 1997; Wilmut et al., 1997; Cognie, 1999). To obtain large numbers of oocytes (eggs) or embryos from ewes, the method of inducing follicular development with follicle stimulating hormone (FSH) is widely used (Gordon, 1997, Cognie, 1999). The ewe naturally releases FSH from the anterior pituitary gland in response to gonadotropin releasing hormone (GnRH) to promote follicular development. Injected into ewes for two or more days at regular intervals, FSH promotes development of a large number of follicles on each ovary (Jablonka-Shariff et al., 1994, 1996). The aim of this study was to determine the effects of FSH on the number of follicles, the recovery and quality of oocytes, the ability of these oocytes to undergo in vitro fertilization, and ovulation rates over time from ewes synchronized with norgestomet (a synthetic progestogen).

MATERIALS AND METHODS

Ewes of mixed breeds were used for these experiments during the reproductive season of fall and winter 1998. For experiments 1 and 2, ewes were implanted in the left ear with Synchro-Mate-B (1/2 implant; SMB; Merial Limited, Athens, GA) in the morning and left in place for 14 days. SMB contains

norgestomet, a potent synthetic progestin that provides high blood levels of progestin which in turn prevents follicular development, thereby synchronizing estrus upon removal of the implant. On day 14 (day 0 = day of SMB implantation), SMB implants were removed through a small incision made in the skin at the distal end of the implant.

Experiment 1, part A: Induction of multiple follicular growth and oocyte collection.

Ewes (n = 29) were randomly distributed to three groups which were given one of three treatments: no treatment (control, n = 10), FSH injected for two days (2D, n = 9) or 3 days (3D, n = 10). Beginning on the morning of Day 12 (3D group) or Day 13 (2D group) after SMB implantation, ewes received twice daily (morning and evening) intramuscular injections of FSH (porcine FSH with 10% luteinizing hormone; Sioux Biochemical, Sioux Center, IA). Injections were as follows: Day 1, 5 units (1.0 ml)/injection; Day 2, 4 units (0.8 ml)/injection; Day 3, 3 units (0.6 ml)/injection (total dose: 2 day treatment = 18 units; 3 day treatment = 24 units). SMB was removed on Day 14 and a laparotomy was performed on Day 15 at 15 hours after the removal of the SMB implant, in order to count follicles and retrieve oocytes.

At laparotomy, the ovaries were exteriorized and the number of follicles were counted on each ovary. In addition, for each follicle, the surface diameter was measured and follicles were classified as <3mm (small), 3-8mm (medium), and >8mm (large) before oocyte collection. Oocytes were then collected using a 22-gauge 1-inch needle and a syringe containing approximately 0.2 ml of collection media that consists of TCM-199 (Sigma, St. Louis, MO), 2% heat-inactivated fetal bovine serum (FBS; Gibco, Gaithersburg, MD), heparin (Sigma), and penicillin/streptomycin (Watson et al., 1994). Each collected follicle was washed/flushed three times with the collection media. The media and follicular fluid from each follicle was placed into separate petri dishes.

By using a stereoscope, each dish was searched and the recovered oocyte(s) was transferred to a petri dish with fresh collection media at which point all oocytes from individual ewes were combined. Oocytes were then evaluated based on morphology and categorized as healthy or atretic according to Thompson et al. (1995). All oocytes were washed three times in maturation media before being transferred into stabilized maturation media (TCM-199, 10% FBS, ovine FSH [oFSH-RP-1; NIAMDD-NIH, Bethesda, MD], ovine LH [oLH-26; NIADDK-NIH], estradiol [Sigma], glutamine [Sigma], sodium pyruvate [Sigma], and penicillin/streptomycin; Watson et al., 1994).

Experiment 1, part B: In vitro fertilization of collected oocytes.

Oocytes collected from ewes in part A were subjected to in vitro fertilization (IVF) and evaluated for fertilization rates. The oocytes were matured for 21-24 hours at 39^0 C, 5% CO_2, and 95% air. After maturation procedures, the oocytes were again evaluated for health based on morphology. Oocytes classified as healthy were separated and used for in vitro fertilization. The cumulus cells were removed by a 1% hyaluronidase (Type I-S; Sigma) treatment and the healthy oocytes were transferred to

stabilized fertilization media, consisting of synthetic oviductal fluid (SOF; Tervit et al., 1972) and 2% heat inactivated sheep serum collected from sheep on day 3 of the estrous cycle (O'Brian et al., 1997).

Frozen semen, which was pooled from 4 NDSU rams, was thawed and viable sperm were separated using the swim up technique (Yovich, 1995). In the swim up technique, the healthy and viable sperm from a semen fraction swim into the media (Modified Sperm Washing Medium, Irvine Scientific, Santa Ana, CA) which lays on top of the thawed semen pool. This media containing the motile healthy sperm is then centrifuged, counted and used for in vitro fertilization. 0.5-1.0 x 10^6 sperm/ml was added to the oocytes (up to 20 oocytes/500 µl/well). The oocytes were incubated with the sperm for 17-20 hours at which time the embryos were washed three times with culture media without glucose (SOF supplemented with BSA, glutamine, MEM amino acids, BME amino acids [Sigma], and penicillin/streptomycin; Catt et al., 1997). The dishes were evaluated, 48-60 h after adding sperm to the oocytes, to determine the number of cleaved oocytes (i.e., embryos). Unfortunately, IVF data from all ewes is not reported herein because of a semen pool of poor quality used for IVF procedures at the beginning of this study. Rather, IVF data reported herein is only from oocytes subjected to IVF procedures from a second semen pool.

Experiment 2: Timing of ovulation.

This study was designed to determine when follicles would naturally ovulate in ewes treated with FSH. Starting on Day 12 of SMB implant, ewes (n = 16) received twice daily (morning and evening) intramuscular injections of FSH for four days as follows; Day 12, 5 units (1.0 ml)/injection; Day 13, 4 units (0.8 ml)/injection; Day 14 and 15, 3 units (0.6 ml)/injection (total dose = 30 units). SMB was removed on Day 14.

At 36, 48, and 60 hours after SMB removal, ewes were subjected to a laparoscopic examination to determine ovulation rates. Both ovaries were observed and the number of corpora hemorrhagica (CH), which represent recently ovulated follicles were counted. Six days after the last laparoscopic observation, ewes were subjected to a laparotomy. The number of corpora lutea (CL) on each ovary were counted. Ovulation rates (%) were then calculated for 36, 48, and 60 hours by dividing the number of CH observed at each time by the total number of CL at the time of laparotomy.

Statistical analysis

All data is reported as means $\pm$ the standard errors.

Experiment 1

Numbers of follicles and oocytes and percentages of matured oocytes and fertilized oocytes for non-treated and FSH-treated ewes were analyzed by using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1985). When the F-test was significant, differences between specific means were evaluated using t-tests. Relationships between treatments in part A were evaluated using Duncan's test. The relationships betwen treatments in part B were evaluated using the Dunn's test.

Experiment 2

The number of CH and ovulation rates at several time points after SMB removal were analyzed using simple linear regression of the Statistical Analysis System (SAS, 1985).

RESULTS

Experiment 1: Part A

Table 1 presents number of small, medium and large follicles on ovaries of non-treated and FSH treated ewes.

Table 1. Number of small, medium and large follicles in non-treated and FSH-treated ewes.

		Number of Follicles			
Treatment	n	<3mm	3-8mm	> 8mm	Total
None	10	3.3±0.83	4.6±0.72 [a]	0.3±0.21	8.2 +0.99 [a]
2D FSH	9	2.0±0.94	13.4 +1.69 [b]	0.7±0.32	16.2 +2.06 [b]
3D FSH	10	1.3±0.60	19.3 +2.06 [c]	0.8±0.42	21.4 +2.04 [c]

[a, b, c] - means ± SEM differ within a column, $p<0.01$.

n - number of ewes.

FSH treatment increased ($p < 0.01$) the number of medium and total number of follicles, but did not affect the number of small or large follicles. The 3D FSH-treatment group had greater ($p<0.01$) numbers of medium follicles and total numbers of follicles than the 2D FSH-treatment group.

Table 2 presents the number of oocytes recovered from small, medium and large follicles.

Table 2. Number of oocytes recovered from small, medium and large follicles for non-treated and

FSH-treated ewes.

		Number of oocytes recovered			
Treatment	n	<3mm	3-8mm	>8mm	Total
None	10	3.1 [a]±0.6	3.9 [a]±0.7	0.2±0.1	6.4 [a]±0.5
2D FSH	9	1.1 [b]±0.6	9.2 [b]±1.4	0.2±0.2	10.1 [a,b]±1.6
3D FSH	10	0.7 [b]±0.4	13.2 [b]±1.8	0.5±0.4	14.4 [b]±1.9

[a, b] - means ± SEM differ within a column, $p<0.02$.

n - number of ewes.

FSH treatment affected ($p<0.02$) the number of oocytes recovered for small and medium but not large follicles. The number of oocytes recovered from medium follicles and the total number of oocytes recovered was greater ($p<0.01$) in FSH-treated ewes than non-treated ewes.

Table 3 presents the total recovery rate and the proportion of healthy and atretic oocytes from non-treated and FSH-treated ewes.

Table 3. Recovery rate, and percentages of healthy and atretic oocytes for non-treated and FSH-treated ewes.

Treatment	n	Total recovery rate (%)*	% of healthy oocytes**	% of atretic oocytes**
None	10	76.1±5.5	67.7 ±5.9 [a]	32.3±5.9
2 day FSH	9	62.1±6.1	87.3±5.6 [b]	12.7±5.6
3 day FSH	10	61.0±7.1	95.1±2.3 [b]	4.9±2.3

[a, b]- means ± SEM differ within a column , $p<0.05$.

n - number of ewes.

*Calculated by dividing the number follicles flushed by the number of oocytes found X 100.

**Percent healthy or atretic oocytes of the total oocytes recovered.

Recovery rate was similar across all treatment groups. The overall proportion of healthy oocytes was higher ($p<0.01$) in FSH-treated ewes than non-treated ewes.

Experiment 1: part B

The number and percentage of oocytes used for IVF and the number and percent of oocytes cleaved in non-treated and FSH-treated ewes is presented in Table 4.

Table 4: Number of oocytes used for in vitro fertilization and cleavage rate of oocytes from non-treated and FSH-treated ewes.

Treatment	n	# of oocytes used for IVF	% of oocytes used for IVF*	# of oocytes cleaved	Rate of cleavage (%)**
None	5	4.4 ± 1.3^{a}	63.8 ± 12.6	3.0 ± 1.0	67.6 ± 9.6^{c}
2D FSH	4	10.5 ± 2.6^{b}	84.4 ± 4.7	6.5 ± 1.5	70.5 ± 13.7^{c}
3D FSH	7	10.4 ± 2.0^{b}	85.3 ± 6.1	4.7 ± 1.3	42.3 ± 5.9^{d}

^{a,b,c,d} - means $\pm$ SEM differ within a column, $p<0.02$.

n - number of ewes.

* Calculated by dividing the number of healthy oocytes used for IVF by the number recovered X 100.

** Calculated by dividing the number of oocytes cleaved by the number of oocytes used for IVF X 100.

The number of healthy oocytes used for IVF was higher ($p<0.02$) in FSH-treated than non-treated ewes. However, the percent of healthy oocytes used for IVF and the number of oocytes cleaved after IVF procedures was similar for all treatments. The cleavage rate was lower ($p<0.01$) for the 3D FSH-treated group than for the non-treated ewes or the 2D FSH-treated group.

Experiment 2

Experiment 2 was designed to determine timing of ovulation in ewes treated with FSH. Number of ovulations and ovulation rates are presented in Table 5.

Table 5: Number of ovulations and ovulation rates at several time points after SMB removal in FSH-treated ewes.

Time	n	# of CH	Ovulation Rate (%)*
36 Hours	16	0.63 ± 0.26^{a}	3.99 ± 1.7^{a}
48 Hours	13	8.69 ± 1.74^{b}	53.6 ± 7.7^{b}
60 Hours	16	10.69 ± 1.59^{c}	66.8 ± 6.2^{c}
Laparotomy	16	17.31 ± 2.46^{d}	100^{d}

^{a,b,c} -means $\pm$ SEM differ with in column, $p<0.01$.

d - not used in statistical analysis.

n - number of ewes.

*Calculated by dividing of the number of CH observed at laparoscopy by the total number of CL observed at time of laparotomy X 100.

The number of CH and the ovulation rate differed ($p<0.01$) among different time points. The number of CH increased from 0.63 to 10.7, and the ovulation rate increased from 4% to 67% from 36 to 60 hours after SMB removal.

Figure 1 shows the linear relationship of ovulations over 3 time points in FSH-treated ewes.

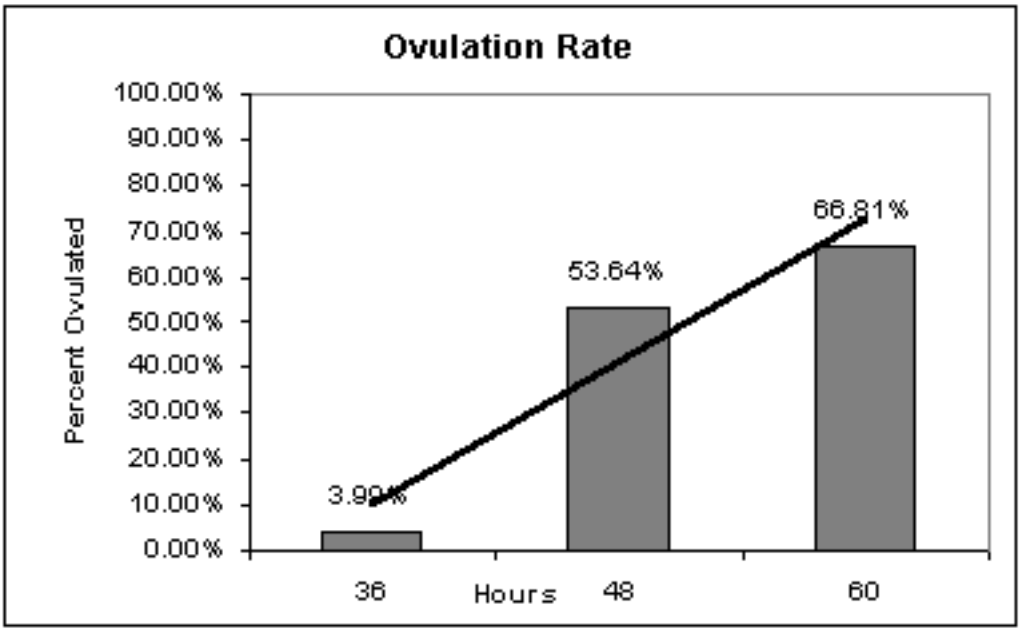

Figure 1: Ovulation rate at several time points in FSH-treated ewes.

The trendline represents the rate of ovulations over the 60 hour period. Both the number of CH and the ovulation rate increased linearly (R^2 = 0.39 and 0.58, respectively, $p < 0.01$) from 36 to 60 hours after SMB removal (Figure 1). At 48 hours, 54% of the follicles ovulated and at 60 hours 67% have ovulated. However, approximately 33% still remained unovulated 60 hours after implant removal.

DISCUSSION

As discussed earlier, assisted reproductive technologies are powerful tools in the animal industry for genetic improvement and also for enhancing reproductive efficiency. In order for ART methods to be efficient, the number of follicles and the number of oocytes and embryos obtained from animals must be maximized. Unfortunately, less than optimal protocols for induction of multiple follicular growth and superovulation are currently used in the animal industry (Redmer et al., 1998). Along with improving these techniques, oocyte retrieval, maturation, and in vitro fertilization techniques, need improvement.

In the present study, follicular growth in ewes was induced by FSH and synchronized with SMB. Most studies use other methods for synchronization, such as CIDR®, intravaginal sponges, or medroxyprogesterone acetate (Gordon, 1997). However, in the United States, these other products have not been commercially available or are not particularly reliable for these procedures. Please note that SMB used in this report is approved only for use in cattle.

In the present study of FSH induced follicular development, the total number of follicles from ewes treated with FSH for 2 days was 16, and for 3 days was 21. In addition, non-treated ewes had the smallest number of follicles with an average of 8. These data support other reports that FSH treatments are an effective way to induce follicular development in ewes (Jablonka-Shariff et al., 1994, 1996; Gordon, 1997).

For ART, multiple follicular stimulation is only effective if multiple oocytes or embryos can be collected. In our study, the majority of the oocytes were collected from medium sized follicles. We did recover over half of the oocytes from the follicles that were less than 3 mm. However, it was more difficult to aspirate oocytes from small follicles because the needles used for collecting were often too large for the follicle and flushing with media was difficult. Baldassarre et al. (1994) reported that it was easier to collect oocytes from follicles smaller than the 5 mm. They found that the larger follicles were difficult to aspirate because the contents appeared more dense and the large follicles tended to 'explode.' We avoided these problems by using media containing heparin and by puncturing each follicle through the ovarian stroma. In our experiment it was easier to collect oocytes from the medium and large follicles. In addition, in FSH-treated ewes the medium follicles constituted 80-90% of the total follicles. Collecting oocytes from these follicles is essential, and consistently high recovery rates is critical to maximize efficiency of these techniques.

Along with consistently high recovery rates, successful in vitro maturation and in vitro fertilization must be achieved. Determining how superovulation techniques affect the quality of oocytes can improve these techniques. Part A of experiment 1 showed that the number of healthy oocytes was higher in FSH-treated ewes than in non-treated ewes. However, part B of experiment 1 demonstrates that the percent of healthy oocytes after maturation was not significantly different among treatment groups, but tended to be higher in the FSH-treated ewes than the non-treated ewes. If the number of animals in this study was higher, perhaps, statistically we would have seen a difference.

The percent of oocytes cleaved was higher in the non-treated ewes and the ewes treated with FSH for 2 days than ewes treated with FSH for 3 days. The cleavage rate for ewes treated with FSH for 3 days was only 42%. The non-treated ewes and ewes treated with FSH for 2 days had a cleavage rate of approximately 70%. This is similar to the 68% cleavage rate reported by Slavik et al. (1992) and 72% by Ledda et al. (1997). However, O'Brien et al (1996, 1997), and Watson et al. (1994) reported cleavage rates of about 80%. Again, increasing cleavage rates through the use of appropriate follicular induction techniques only prove to be a key factor in the overall efficiency of ART.

Experiment 2 was designed to determined the ovulation rate over time in ewes that were synchronized with SMB and stimulated with FSH. Between 36 hours and 48 hours after SMB removal, 50% of the ovulations occurred, with an additional 13% occurring between 48 and 60 hours. However, it is interesting to note that 33% of the follicles had not ovulated by 60 hours. Previous studies show that the optimal time to inseminate is between 54 and 60 hours after progestin removal (Findlater et al., 1991). Our data shows that more research needs to be conducted to determine the optimal time for inseminating ewes that are treated with SMB and FSH.

The results of this and future studies will ultimately lead to improved and efficient methods for obtaining large numbers of high quality oocytes and embryos for transfer programs. Improvement in these techniques will enhance the overall efficiencies of ART for farm use.

ACKNOWLEDGEMNTS

We greatfully acknowledge Jim Kirsch, Kim Kraft, Tim Johnson, and Wes Limesand for expert technical assistance.

LITERATURE CITED

Baldassarre, H., de Matos, D. G., Furnus, C.C., Castro, T.E., and E.I. Cabrera Fischer. 1994. Technique for efficient recovery of sheep oocytes by laparoscopic folliculocentesis. Anim. Reprod. Sci. 25: 145-150.

Catt, S.L., O'Brien, J.K., Maxwell, W.M.C. and G. Evans. 1997. Effects of rate of development of in vitro-produced ovine embryos on sex ratio and in vivo survival after embryo transfer. Theriogenology 48: 1369-1378.

Cognie, Y. 1999. State of the Art in sheep-goat embryo transfer. Theriogenology. 51:105-116.

Findlater, R.C.F., Haresign, W., Curnock, R.M. and Beck, N.F.G. 1991. Evaluation of intrauterine insemination of sheep with frozen semen: effects of time of insemination and semen dose on conception rates. Anim. Prod. 53: 89-96.

Gordon, I. Controlled Reproduction in Sheep and Goats, CAB International, 1997.

Holm, P., Walker, S.K., and R.F. Seamark. 1996. Embryo viability, duration of

gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. J. Reprod. Fertil. 107:175-181.

Jablonka-Shariff, A., L.P. Reynolds and D.A. Redmer. 1996. Effects of gonadotropin treatment and withdrawal on follicular growth, cell proliferation, and atresia in ewes. Biol. Reprod. 55:693-702.

Jablonka-Shariff, A., Fricke, P.M., Grazul-Bilska, A.T., Reynolds, L.P., and D.A. Redmer, 1994. Size, number, cellular proliferation, and atresia of gonadotropin-induced follicles in ewes. Biol. Reprod. 51:531-540.

Ledda, S., Bogliolo, L., Calvia, P., Leoni, G., and S. Naitana. 1997. Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. J. Reprod. Fertil. 109: 73-78.

O'Brien, J.K., Dwarte, D., Ryan, J.P., Maxwell, W.M.C. and G. Evans. 1996. Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. Reprod. Fertil. Dev. 8: 1029-1037.

O'Brien, J.K., Catt, S.L., Ireland, K.A., Maxwell, W.M.C. and G. Evans. 1997. In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. Theriogenology 47: 1433-1443.

Redmer, D.A., J.D. Kirsch, K.C. Kraft, W. Limesand, A.T. Grazul-Bilska and L.P.

Reynolds. 1998. Use of a synthetic progestogen in combination with a superovulatory treatment for induction of synchronized estrus in seasonally anovular ewes. 39th Annual Western Dakota Sheep Day, Hettinger, ND, Report 39:62-66.

SAS. 1985. User's Guide, Statistics, 5th Edn., Statistical Analysis System Inst., Cary, NC.

Schnieke, A. E., Kind, A.J., Ritchie, W. A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A., and K.H.S. Campbell. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278: 2130-2133.

Slavik, T., Fulka, J., and I. Goll. 1992. Pregnancy rate after the transfer of sheep embryos originated from randomly chosen oocytes matured and fertilized in vitro. Theriogenology 38: 749-756.

Tervit, H.R., Whittingham, D.G., and L.E.A. Rowson. 1992. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30: 493-497.

Thompson, J.G., Gardner, D.K., Pugh, P.A., McMillan, W.H., and H.R. Tervit. 1995. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol. Reprod. 53: 1385-1391.

Watson, A.J., Watson, P.H., Warnes, D., Walker, S.K., Armstrong, D.T., and R.F. Seamark. 1994. Preimplantation development of in vitro-matured and in vitro-fertilized ovine zygotes: comparison between coculture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere. Biol. Reprod. 50: 715-724.

Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. S. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature. 385: 810-813.

Yovich, J.L. Gametes - The Spermatozoon. J.G. Grudzinskas (ed.) & J.L. Yovich (ed.) Cambridge University Press. 1995.