Acta Veterinaria Hungarica 51 (1), pp. 95–101 (2003)

IN VITRO FERTILISATION OF *IN VIVO* MATURED PORCINE OOCYTES OBTAINED FROM PREPUBERAL GILTS AT DIFFERENT TIME INTERVALS AFTER hCG INJECTION

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(Received May 7, 2002; accepted September 11, 2002)

The goal of the present study was to find out the best interval after hCG injection in PMSG primed prepuberal gilts for retrieval of *in vivo* matured oocytes for *in vitro* fertilisation (IVF). Altogether 66 gilts were superovulated with 1500 IU PMSG and 500 IU hCG 72 h later. Ovum pick up was performed endoscopically 24, 28, 32 or 36 h after hCG and a total of 869 cumulus-oocyte-complexes (COCs) were aspirated from 1400 follicles. COCs were tested for quality, and an aliquot was immediately fixed and stained to determine meiotic configuration. The remaining COCs were fertilised *in vitro* using frozen-thawed epididymal semen. Quality and developmental stage of embryos were tested after IVF, and the number of nuclei was counted. At 24 to 32 h after hCG only few oocytes have entered the second meiotic cycle (18 to 25% vs. 58% at 36 h, $p < 0.05$). The overall cleavage rate was significantly influenced by insufficient maturation rate at the early collection times (14% at 24 h vs. 49% at 36 h). Additionally, when oocytes were collected 24 to 32 h vs. 36 h the cleavage rate based on mature oocytes was lower (26 vs. 62%, $p < 0.05$). Once embryonic development has been initiated, the further *in vitro* development to blastocyst stages did not differ between groups. However, the number of cells was lower at collection times 24 to 32 h as compared to 36 h after hCG (12 to 15 cells vs. 22 cells, $p < 0.05$). The results indicate that the time of COC collection affects the *in vitro* developmental competence up to the blastocyst stage and should not be performed earlier than 36 h after hCG treatment.

Key words: Oocyte, *in vivo* maturation, *in vitro* fertilisation, ovum pick up, swine

Currently there is an increasing interest to yield viable pig embryos by *in vitro* techniques and to produce transgenic pigs for biomedical and basic research purposes. The main source of oocytes for *in vitro* maturation and fertilisation are antral follicles of slaughtered pigs. Selected oocytes with uniform ooplasm and compact cumulus cell layers may undergo nuclear maturation up to 90% if they

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0236-6290/2003/$ 20.00

were incubated in appropriate maturation media (Niwa, 1993). However, *in vitro* maturation influences nuclear and cytoplasmic maturation of porcine oocytes, and subsequent pronuclear formation, first cleavage and blastocyst formation (Laurincik et al., 1994; Rath et al., 1995; Funahashi and Day, 1997).

Difficulties of *in vitro* maturation can be overcome if oocytes are derived from mature preovulatory follicles by means of endoscopic ovum pick up (Brüssow and Rátky, 1994). Under physiological conditions, oocytes undergo a final maturation before they are released from the follicles at about 40 to 44 h after the LH peak. This process can be mimicked in prepuberal gilts by treatment with 1000 to 2000 IU pregnant mare serum gonadotropin (PMSG) and 500 IU human chorionic gonadotropin (hCG) 72 h later. Ovulation can be expected in a constant time interval after hCG treatment.

The goal of the present study was to find the optimal interval for oocyte retrieval after hCG treatment based on oocyte maturation, fertilising competence and embryo development under *in vitro* conditions.

Materials and methods

Gilts

Altogether, 66 prepuberal crossbred Landrace gilts at the age of 175–180 days and body weight of 90 kg were used. Follicular development was stimulated by treatment with 1500 IU PMSG (Pregmagon®, Impfstoffwerk Dessau-Tornau, Germany) and LH surge was mimicked with 500 IU hCG (Choriolutin®, Albrecht, Aulendorf, Germany) 72 h later.

Oocyte retrieval

Gilts were anaesthetised with ketamine/xylazine (Ursotamin® and Xylazin®, Serumwerk Bernburg, Germany). Cumulus-oocyte-complexes (COCs) were aspirated by endoscopic ovum pick up as described by Brüssow and Rátky (1994), 24, 28, 32 or 36 h after hCG. Follicular aspiration was performed with a two-way cannula (40 mm length, 16-gauge) attached to an electric aspiration pump (model 3014, Labotect, Göttingen, Germany). The initial vacuum was set to 100 mm Hg, corresponding to a volume of 17 ml/min. The tip of the aspiration cannula was inserted into a follicle, the follicular content was aspirated, then the follicle was refilled with heparinized PBS and aspirated again. Only macroscopically healthy, well-vascularised and translucent follicles with a diameter of > 5 mm were punctured. Aspirated COCs were tested for quality under a stereomicroscope and an aliquot was immediately fixed in acetic acid and stained with Aceto-Orcein 24 h later for cytogenetic analysis.

In vitro *fertilisation and culture*

The remaining COCs were fertilised *in vitro* using frozen-thawed epididymal semen. Therefore, immediately after slaughter epididymides of the three boars were removed and spermatozoa were flushed with Androhep extender from the ductus deferens in caudal direction into the cauda epididymis. The collected semen was centrifuged for 10 min at 800 *g* and the pellets were resuspended with cooling extender to give a sperm concentration of 3×10^8 sperm cells per ml (Rath and Niemann, 1997). Within two hours the semen was cooled to +5 °C and the final sperm concentration was adjusted to 2×10^8 sperm cells per ml using a freezing extender. Final glycerol concentration was 2%. Semen samples were immediately filled into 0.25 ml plastic straws (Minitüb system, Minitüb, Tiefenbach, Germany) containing 5×10^7 sperm cells and were frozen in nitrogen vapour 4 cm above the liquid nitrogen level. After 20 min the straws were plunged into liquid nitrogen and stored until use. Shortly before *in vitro* fertilisation the straws were thawed in water bath (38 °C, 20 sec). After estimation of the motility under a phase contrast microscope at 200× magnification semen samples were centrifuged at 800 *g* for 3 min and the sperm pellet was resuspended with fertilisation medium TALP.

For IVF, five oocytes were cultured in each microdrop (40 µl) covered with silicone oil (DC 200; Serva, Heidelberg, Germany) together with 10 µl of the semen sample in a humidified atmosphere at 39 °C and 5% CO_2. Eighteen hours after the initiation of fertilisation the potential zygotes were transferred into 2 ml culture medium (NCSU 23). At 48, 72 and 120 h after IVF embryos were tested microscopically for quality and developmental stage. On the last day of culture embryos were stained with Hoechst 33342 in order to count the number of nuclei.

Statistical methods

Results were analysed by one-way analysis of variance and pair-wise multiple comparison procedure (Tukey test). P-values < 0.05 were considered to be statistically significant.

Results

In total 1400 follicles were punctured and 869 COCs aspirated (overall recovery rate 62.1%). Results of follicular puncture and oocyte recovery are presented in Table 1.

The number of developed follicles, the number of aspirated COCs and the recovery rate did not differ between groups at different time intervals after hCG.

Differences were obtained according to the oocyte maturation rates (Table 2).

Table 1

Number of punctured follicles and aspirated COCs at different collection times after hCG

		Hours after hCG			
		24	28	32	36
Gilts	n	15	12	16	23
Follicles punctured	mean ± SD	21.3 ± 9.7	21.3 ± 8.8	19.3 ± 8.8	20.7 ± 11.9
COCs aspirated	mean ± SD	13.7 ± 5.5	15.8 ± 8.3	11.3 ± 6.0	11.8 ± 8.5
Recovery rate (%)	mean ± SD	69.0 ± 18.1	72.8 ± 14.2	60.0 ± 23.1	57.4 ± 22.8

Table 2

Chromatin configuration of oocytes at different collection times after hCG

Time	Germinal vesicle		Meta- Ana- Telophase I		Metaphase II	
	Mean	± SD	Mean	± SD	Mean	± SD
24 h	37.1	4.0	47.7[a]	6.6	17.8[a]	7.5
28 h	20.1	25.1	56.6[a]	31.2	23.3[a]	6.1
32 h	18.0	11.7	56.7[a]	13.2	25.3[a]	5.6
36 h	27.9	5.8	11.9[b]	4.0	57.8[b]	11.4

[a:b]within a row $p < 0.05$

Between 24 and 32 h after hCG most of the oocytes (75 to 85%) were at the immature stage (Germinal Vesicle) or in maturation (Meta- to Telophase I). More oocytes were matured (Metaphase II) at 36 h-collection time compared to the other one ($p < 0.05$).

After IVF the cleavage rate was influenced by collection time after hCG ($p < 0.05$). Results of *in vitro* fertilisation are shown in Table 3.

Table 3

Results of *in vitro* fertilisation (cleavage rate) at different collection times after hCG

Time	Overall cleavage rate (%)		Cleavage rate (% of all matured oocytes)	
	Mean	± SD	Mean	± SD
24 h	13.6[a]	14.8	25.8[a]	24.4
28 h	30.2	18.6	47.7	23.5
32 h	34.8	17.3	55.6[b]	25.0
36 h	48.8[b]	21.3	61.9[b]	17.9

[a:b]within a row $p < 0.05$

The overall cleavage rate of oocytes was influenced by the insufficient maturation rate at the early collection times (24 to 32 h: 18 to 25% Metaphase II vs. 58% at 36 h). Additionally, referred to the number of matured oocytes, cleavage rates were higher ($p < 0.05$) at the late collection times.

Once embryonic development has been established, further *in vitro* development to blastocyst stage seems not to be different between groups (Table 4). However, the number of cells of blastocysts was significantly affected by collection time. The highest number was observed at 36 h compared to 24 to 32 h ($p < 0.05$).

Table 4

Results of *in vitro* embryo development

Time	Blastocyst formation rate (% of cleaved ova)		Blastocyst cell number	
	Mean	± SD	Mean	± SD
24 h	45.8	41.9	12.2[a]	2.5
28 h	21.0	24.8	15.3[a]	4.3
32 h	10.0	22.4	14.0	0
36 h	53.9	19.8	21.8[b]	5.4

[a:b]within a row $p < 0.05$

Discussion

Endoscopic ovum pick up is a suitable method to recover *in vivo* matured porcine cumulus-oocyte-complexes from preovulatory follicles. The oocyte recovery rate achieved here in prepuberal gilts (62%) was comparable to those reported for cycling Landrace and Mangalica gilts (60 to 72% and 54%; Brüssow and Rátky, 1994; Brüssow et al., 1997; Torner et al., 1998; Egerszegi et al., 2001).

Follicular maturation and oocyte maturation are highly correlated (Xie et al., 1990; Torner et al., 1998). In our study the percentage of meiotically matured oocytes increased in relation to the collection times after hCG from 18% (24 h) to 58% (36 h). In cycling gilts 41% (22 h) and 70% (34 h) of the oocytes were matured (Torner et al., 1998).

Cleavage rate after *in vitro* fertilisation increased in relation to oocyte collection after hCG from 14 to 49%. Rath (1992) and Rath et al. (1995) reported on 32 to 42% cleavage after *in vitro* fertilisation of *in vivo* matured oocytes recovered 34 to 36 h after hCG. Forty-six percent of *in vivo* matured oocytes cleaved to the 2–4-cell stage in culture compared to 3% of *in vitro* matured ova (Nagashima et al., 1996). Although culture conditions for *in vitro* maturation and fertilisation have been improved continuously, the developmental competence of *in vitro* derived oocytes is still low (range between 3 and 36%; Funahashi and

Day, 1997; Abeydeera, 2001). *In vivo* derived embryos, however, developed *in vitro* at 77 to 85% to blastocysts whereas only 50% of *in vivo* matured but *in vitro* fertilised oocytes reached the blastocyst stage (Rath, 1995). The development to blastocysts was lower after maturation and fertilisation *in vitro* (11 vs. 42%, Nagashima et al., 1996). In our study there was a tendency of increasing blastocyst formation in relation to the time of oocyte collection ($p = 0.08$) and the highest blastocyst formation rate (54%) was achieved from oocytes recovered 34 h after hCG.

Although *in vitro* fertilised oocytes develop to the blastocyst stage, their low cell number is still a problem. Blastocysts derived 24 h after hCG had a lower number of cells compared to 36 h (12 vs. 22%, $p < 0.05$). In the study of Macháty et al. (1998) embryos developed well to the blastocyst stage in NCSU23 medium; however, *in vitro* derived blastocysts had a lower cell number as compared to their *in vivo* counterparts (28 vs. 59).

In conclusion, the results clearly show that *in vivo* matured and *in vitro* fertilised oocytes derived from PMSG/hCG treated prepuberal gilts have a high competence to develop to blastocysts. However, their competence is effected by the collection time. *In vivo* matured oocytes should not be collected earlier than 36 h after the LH peak simulated by hCG injection.

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