60, 1657–1663]) 6–7 h after insemination and cultured for 65–66 h. Embryos which had cleaved by 72 h post insemination were further cultured 96 h in CDM-2 (Zhang M *et al*. 2003 Theriogenology 60, 1657–1663) containing 0.12 IU insulin mL^{-1}. Cleavage and blastocyst rates per oocyte inseminated were recorded on Day 3 and Days 7–8 after insemination, respectively. Data were analyzed by ANOVA procedures with replicates and treatments in the model. There was no significant difference in cleavage rate or blastocyst rate between X and Y sperm treatments. These results indicate that embryos produced with Y sperm do not reach the blastocyst stage in significantly higher proportions than embryos produced with X sperm in this chemically defined medium + FAF-BSA. Apparently, this IVC system leads to a more synchronous development of male and female embryos than other methods of producing bovine embryos *in vitro*.

Table 1. Cleavage and blastocyst rates with X and Y sperm

Sperm type	Oocytes (*n*)	Cleaved[a] (%)	% Blastocysts/ oocyte[a]	% becoming blastocysts[a] Day 7	Day 8
X	611	50.3 ± 4.3	19.9 ± 4.3	76.5 ± 5.9	23.5 ± 5.9
Y	753	57.6 ± 7.6	21.0 ± 2.5	77.1 ± 2.6	22.9 ± 2.6

[a] No significant difference, $P > 0.05$: LS means ± SE.

This research was supported by XY, Inc., Fort Collins, CO, USA.

Sperm Injection

313 EFFECTS OF ETHANOL TREATMENT AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI) ON SPERM ASTER FORMATION AND THE MICROTUBULE ORGANIZATION OF BOVINE OOCYTES

N. Fujinami[A], *Y. Hosoi*[A,B], *H. Kato*[A], *T. Mitani*[A], *K. Matsumoto*[A,B], *K. Saeki*[A,B], *and A. Iritani*[A,B]

[A]Institute of Advanced Technology, Kinki University; [B]Department of Biology-Oriented Science and Technology, Kinki University, Wakayama, Japan. Email: hosoi@gene.waka.kindai.ac.jp

The cleavage rate of bovine embryos is very low without activation of oocytes after intracytoplasmic sperm injection (ICSI), although both male and female pronuclei are formed. We previously reported that the stimulus due to the injected sperm alone was sufficient to lower the MPF activity of bovine oocytes after ICSI, and the activation treatment of oocytes with ethanol at 4 h after ICSI served to maintain the low levels of MPF activity until the next cell cycle started (Fujinami *et al*. 2004 J. Reprod. Dev. 50, 171–178). These results suggested that activation treatment is necessary to improve the embryonic development after bovine ICSI. In bovine fertilization, the sperm introduces the centrosome into the oocyte. The centrosome acts as the microtubule-organizing center and microtubules are organized within the oocyte. It is reported that the sperm aster is important for the normal fertilization process. Therefore, failure of sperm aster formation possibly causes the failure of cleavage following fertilization. To investigate the reason of the low cleavage rate after bovine ICSI without artificial activation treatment, we examined sperm aster formation and the microtubule organization in bovine oocytes with or without activation treatment after ICSI. Bull spermatozoa immobilized by piezopulse was injected into bovine oocytes matured *in vitro*. At 4 h after ICSI, oocytes were treated with 7% ethanol in TCM199 for 5 min for activation. Oocytes were fixed at 6 and 12 h after ICSI, and the microtubule organization was examined by using specific antibodies and immunofluorescence microscopy. The cleavage rate (51% vs. 15%) and the developmental rate to the blastocyst stage (13% vs. 3%) were increased by ethanol treatment after ICSI (with or without ethanol treatment, respectively, $P < 0.05$). In oocytes activated with ethanol after ICSI, both the sperm aster formation rate at 6 h and the microtubule organization rate at 12 h after ICSI were significantly higher than in oocytes without activation treatment (58%, 80% vs. 12%, 26%, $P < 0.05$). It was reported that the sperm aster has an important role for the pronuclear movement to make the male and female pronuclei come into close apposition. From these results, it was concluded that oocyte activation after bovine ICSI promoted sperm aster formation and microtubule organization, and was effective to improve embryonic development.

This study was supported by a Grant-in-Aid for the 21st Century COE Program of the Japan MEXT, and by a grant from the Wakayama Prefecture Collaboration of Regional Entities for the Advancement of Technological Excellence of the JST.

314 FULL-TERM DEVELOPMENT OF RAT OOCYTES MICROINSEMINATED WITH FREEZE-DRIED SPERMATOZOA

M. Hirabayashi[A], *M. Kato*[A], *and S. Hochi*[B]

[A]National Institute for Physiological Sciences, Okazaki, Japan; [B]Faculty of Textile Science and Technology, Shinshu University, Nagano, Japan. Email: mhirarin@nips.ac.jp

Since freeze-dried spermatozoa can be stored at ambient or refrigerated temperature, the costs required for maintenance and shipping of spermatozoa can be reduced. To date, viable offspring in mice (Wakayama and Yanagimachi 1998 Nat. Biotech. 16, 639) and rabbits (Liu *et al*. 2004 Biol. Reprod. 70, 1776) have been produced by intracytoplasmic sperm injection (ICSI) using freeze-dried samples. The objectives of the present study were to

examine whether freeze-dried rat spermatozoa can participate in full-term development by ICSI, and whether sonication prior to freeze-drying of the spermatozoa influences the offspring rate. Spermatozoa from cauda epididymides of Sprague-Dawley (SD) rats were collected in 10 mM TRIS/HCl buffer supplemented with 50 mM NaCl and 50 mM EGTA. A 2 × 3 factorial-designed experiment was conducted. The sperm suspensions were either sonicated for 10 s using a 10% power output from an ultrasonic cell disruptor or not sonicated. The sperm suspensions were then processed for freeze-thawing (100-μL sample in 1.0-mL cryotube was cooled in liquid nitrogen vapor, stored at −196°C for 48 h, and thawed in a 25°C water bath) and freeze-drying (100-μL sample in 1.5-mL polypropylene tube was frozen in liquid nitrogen for 20 s, lyophilized for 6 h by a freeze-drying apparatus, stored at 4°C for 48 h, and rehydrated with 100 μL ultra pure water), or were subjected to immediate use for ICSI. The sperm heads were microinjected into denuded SD oocytes using a piezo-driven micropipette 2–4 μm in diameter, as described previously (Hirabayashi *et al*. 2002 Transgenic Res. 11, 221). The presumptive zygotes were transferred into oviducts of pseudopregnant Wistar female rats. The *in vivo* developmental potential of rat oocytes microinseminated with fresh, freeze-thawed, and freeze-dried spermatozoa is shown in the table below. Viable rat offspring were produced in all six experimental groups, with the offspring rates at 2.5–35.0%. Sonication treatment of rat spermatozoa to induce membrane disruption and tail/midpiece dissociation from the heads was effective in increasing the offspring rate after ICSI. The positive effect of sperm sonication may be explained as facilitating decondensation of sperm heads by membrane disruption in the spontaneously activating rat oocytes. Thus, successful participation of freeze-dried rat spermatozoa into full-term development was demonstrated by applying the ICSI.

Table 1. *In vivo* development of rat oocytes microinseminated with fresh, freeze-thawed, and freeze-dried spermatozoa

Sonication	Fresh control	Frozen-thawed	Freeze-dried
None	6/90 (6.7%)	8/105 (7.6%)	3/119 (2.5%)
+	20/86 (23.3%)	36/103 (35.0%)	11/120 (9.2%)

Offspring/transferred oocytes.

315 AN ATTEMPT AT INDUCING DIFFERENTIATION INTO ROUND SPERMATIDS OF RAT SPERMATOGONIA BY CO-CULTURING WITH SERTOLI CELLS

Y. Iwanami[A], *T. Kobayashi*[A], *M. Kato*[B], *M. Hirabayashi*[B], *and S. Hochi*[A]

[A]Faculty of Textile Science and Technology, Shinshu University, Nagano, Japan; [B]National Institute for Physiological Sciences, Okazaki, Japan. Email: shochi@giptc.shinshu-u.ac.jp

Mammalian spermatogenesis is a complex process of germ cell development at the seminiferous tubules whereby diploid spermatogonia proliferate and differentiate into haploid spermatozoa via round and elongating spermatids in close association with somatic Sertoli cells. In the present study, the potential of rat spermatogonia to undergo meiosis during co-culture with Sertoli cells was assessed. The type-A spermatogonia and Sertoli cells were prepared from Day 7 heterozygous transgenic male rats carrying EGFP DNA, and co-cultured on the dishes (coated; BD Falcon™ 35-3801, or non-coated: BD Falcon™ 35-1008, 4 × 10^6 cells/4-mL dish) at 37°C for 3 days and at 34°C for a subsequent 7 days in 5% CO_2 in air. The culture medium was DMEM medium supplemented with 10% fetal bovine serum, growth factors (10 ng/mL EGF and 10 ng/mL IGF) and various hormones (500 ng/mL FSH, 133 μIU/mL hGH, 5 μg/mL insulin, 0.1 μM testosterone and 0.1 μM dihydrotestosterone). During culture, appearance of round spermatid-like cells (ca. 15 μm in cellular diameter and 7–8 μm in nuclear diameter) was traced. The ploidy of the cells was also analyzed by flow cytometry (FCM). At the end of culture, the proportion of EGFP DNA-bearing cells in the total cultured cell population was examined under UV light at 365 nm. Thereafter, continuation of the spermatid-like cells to full-term development was examined by ooplasmic microinjection (Kato *et al*. 2004 Contemp. Top. Lab. Anim. Sci. 43/2, 13). Briefly, oocytes from the Sprague-Dawley rats were denuded, activated with two direct-current pulses at 100 V/mm for 99 μs and held in 2 mM 6-dimethylaminopurine for 20 min. The nuclei of spermatid-like cells were microinjected into the oocytes by using a piezo impact driving unit, and the injected oocytes after 24 h culture were transferred into recipients. Round spermatid-like cells were first observed at the 5th day of culture on both dishes, but the proportion of spermatid-like cells on the coated dish was higher than that on the non-coated dish. The FCM analysis showed that a single peak of haploid cells was detected in the cell population cultured on the coated dish at the 5th day of culture, while no haploid peak was detected on the non-coated dish. The cultured cells exhibited two distinct patterns of EGFP fluorescence, with a proportion of EGFP-positive cells at 53.5% (total 1,000 counts). The microinsemination into 263 oocytes resulted in the production of 27 oocytes with two pronuclei (10.3%) and 15 cleaved oocytes (5.7%). However, the oviductal transfer of 143 microinseminated oocytes resulted in only 8 implantation sites without viable offspring (5.6%). These results indicated that rat type-A spermatogonial cells seemed to undergo meiosis, but the potential of the cultured spermatid-like cells to participate into full-term development was questionable.

316 SUPPLEMENTAL CYSTEINE PRESENCE DURING THE DECONDENSATION OF SPERM CHROMATIN IMPROVES FERTILIZATION AND BLASTOCYST FORMATION AFTER INTRACYTOPLASMIC SPERM INJECTION IN PIGS

M. Katayama, *T. Cantley*, *A. Rieke*, *and B. Day*

Department of Animal Science, University of Missouri-Columbia, Columbia, MO 65201, USA. Email: hudlowl@missouri.edu

The effect of a cysteine supplement in culture media for oocytes matured *in vitro* after intracytoplasmic sperm injection (ICSI) on fertilization and embryo development were examined. In the first experiment, sperm injected oocytes were cultured in NCSU23 (control) or NCSU23 supplemented

with 0.57–3.71 mM cysteine (0.57–3.71 Cys) for 12 h after ICSI, and then fixed to observe pronuclear formation. In the second experiment, to examine the appropriate duration time of cysteine supplement to support fertilization, sperm-injected oocytes were transferred into NCSU23 following culture in NCSU23 supplemented with 1.71 mM cysteine for 1, 2, 3, 4, 5, 6, or 9 h after ICSI, and then fixed at 12 h. At the same time, morphological changes of sperm heads in oocytes cultured in NCSU23 (1.71 Cys) were observed. In the third experiment, to examine the developmental ability of ICSI embryos fertilized in NCSU23 (1.71 Cys), sperm injected oocytes were cultured under the following conditions for a total of 168 h; NCSU23 (control), NCSU23 (1.71 Cys) for 3 h followed by transfer into NCSU23 (1.71 Cys-3 h), NCSU23 (1.71 Cys) for 12 h followed by transfer in NCSU23 (1.71 Cys-12 h), or NCSU23 (1.71 Cys) (1.71 Cys). Data were pooled from at least five replicates. Values in each replicate were analyzed using one-way ANOVA. Significance of differences was assessed by Student's *t*-test. Culture with several concentrations of cysteine for 12 h showed that 1.71–3.71 Cys significantly ($P < 0.05$) increased fertilization rates above controls or 0.57 Cys (56–60%, 35%, or 48%, respectively). Culture for several duration times with 1.71 Cys showed that fertilization rates increased as the duration time increased to 3 h which was significantly ($P < 0.05$) higher than controls (68% and 34%, respectively), and culture times of greater than 3 h did not increase fertilization rates (58–68%). At 3 h, 59% of oocytes cultured in NCSU23 (1.71 Cys) had decondensed sperm heads and 16% of those had enlarged sperm heads. At 6 h, 50% of oocytes cultured in NCSU23 (1.71 Cys) had male pronuclei. Blastocyst formation rate in 1.71 Cys-3 h was 29% which was higher than for controls (20%). On the other hand, 1.71 Cys-12 h cultures showed low blastocyst formation rates, and continuous culture in NCSU23 (1.71 Cys) for 168 h (1.71 Cys) significantly ($P < 0.05$) decreased blastocyst rates (16% and 7%, respectively). We found that the supplement of 1.71 mM cysteine to NCSU23 for culture of oocytes after ICSI improved fertilization rates. However, the presence of 1.71 mM cysteine for 12 h or longer after ICSI had adverse effects on embryo development. Since 1.71 mM cysteine supplement for 3 h after ICSI improved blastocyst formation with the same fertilization rates as when supplemented for 12 h, the presence of cysteine only during the decondensation of sperm chromatin was found to be associated with the improvement of fertilization and also the promotion of blastocyst formation.

317 FACTORS AFFECTING PRODUCTION EFFICIENCY OF TRANSGENIC RATS BY ICSI-MEDIATED DNA TRANSFER

M. Kato[A], *S. Hochi*[B], *and M. Hirabayashi*[A]

[A]National Institute for Physiological Sciences, Okazaki, Japan; [B]Faculty of Textile Science and Technology, Shinshu University, Nagano, Japan. Email: katomegu@nips.ac.jp

Successful DNA transfer via intracytoplasmic sperm injection (ICSI) was first reported in mice (Perry *et al*. 1999, Science 284, 1180), and was recently extended to rats (Kato *et al*. 2004, Mol. Reprod. Dev. 69, 153). In the present study, factors affecting the production efficiency of transgenic rats by the ICSI-mediated DNA transfer were investigated. Cauda epididymal spermatozoa from Sprague-Dawley rats were sonicated (SO) and/or frozen-thawed (FT) for tail-cutting and membrane-disrupting. The sperm heads were exposed for 1 min to different concentrations (0.02–2.5 ng/μL) of 3.0 kb EGFP DNA solution, and then microinjected into denuded F1 (Donryu × LEW) rat oocytes. The optimal concentration of EGFP DNA was 0.1 ng/μL, as determined by the *in vitro* developmental competence into morulae/blastocysts and the EGFP expression of the ICSI oocytes. The presumptive 1- or 2-cell stage zygotes were transferred into oviducts of pseudopregnant Wistar females, and the presence of EGFP DNA in the offspring was examined by fluorescence under the 480 nm UV light. The production efficiency of transgenic rat offspring was 2.8% (2/71 zygotes transferred), 1.6% (1/63), and 3.3% (2/61) in the oocytes into which SO-, FT-, and SO+ FT-treated sperm heads were injected, respectively. The founder transgenic rats carrying EGFP DNA transmitted the transgenes to their progeny according to the Mendelian fashion (43.8–54.8%), suggesting the stable incorporation of the transgenes into rat genomes. Four rat strains (F344, LEW, Donryu, and Sprague-Dawley) were compared for their suitability as sperm/oocyte donors in the production of transgenic rats by ICSI with SO + FT-treated and 0.1 ng/μL EGFP DNA-exposed sperm heads. The production efficiency of the transgenic rats in the Sprague-Dawley strain (8.2%, 8/98) was significantly higher than that in LEW strain (0.9%, 1/114), while those in F344 (4.3%, 4/92) and Donryu (4.4%, 5/114) strains were intermediate. Attempts were made to introduce three other DNA constructs (5.0 kb plasmid and 208 kb BAC, both with Fyn gene, and 186 kb BAC with Svet1/IRES-Cre gene) into rat genomes by ICSI with SO + FT-treated and 0.1 ng/μL DNA-exposed sperm heads. PCR analysis showed that the Fyn, Fyn/BAC, and Svet1/IRES-Cre DNA constructs were successfully introduced into Sprague-Dawley rat offspring via ICSI, with production efficiencies of 2.8% (3/109), 0.9% (1/109), and 2.4% (3/125), respectively. These results indicate that transgenic rats can be produced by ICSI-mediated DNA transfer using the various types of exogenous DNA and rat strains with different genetic backgrounds.

318 ENHANCEMENT OF FERTILIZATION BY DIGITONIN IN ROUND SPERMATID INJECTION

S. Kishigami, *E. Mizutani*, *S. Wakayama*, *and T. Wakayama*

RIKEN, Center for Developmental Biology, Kobe, Japan. Email: kishigami@cdb.riken.jp

Reproductive technologies allow us to produce offspring using a variety of cells including sperm, spermatids, spermatocytes, somatic cells, and even parthenogenetic oocytes. In each of these technologies, failure of pronuclear formation after injection often prevents successful artificial reproduction. One of the possible causes is assumed to be that the breakage of the cytoplasmic membrane by simple pipetting is not enough to expose the nuclei to the ooplasm for pronuclear formation. To overcome this problem, we applied digitonin, a mild nonionic detergent, for the purpose of the permeabilization of cellular and nuclear membranes before injection. In this study, round spermatid cells in the mouse were used as a model because of their low pronuclear formation rate after injection. First, to examine the permeabilization of spermatids by digitonin, spermatid cells were incubated in CZB medium including 10 μg/mL of digitonin. Interestingly, the spermatids were lysed within 30 s after transfer but not other spermatogenic cells or somatic cells. Next, we conducted round spermatid injection (ROSI) using PVP including digitonin in a similar manner. Spermatids were picked up by injection pipette from spermatogenic cells suspended in a drop of PVP. These spermatids were transferred into another PVP drop including 1 μg/mL or 10 μg/mL of digitonin and left for 30 s. These digitonin-treated spermatids were then directly injected into previously activated oocytes. Six hours after injection, the fertilized oocytes were examined. Pronuclear formation rates were calculated as a proportion of oocytes

with two pronuclei as well as one second polar body to total oocytes with one second polar body (Table 1). After digitonin treatment, fertilization rates significantly increased compared with ROSI without digitonin (Table 1). Further, these fertilized oocytes developed into blastocysts *in vitro* at comparable or higher rates. To further elucidate the effects of digitonin pretreatment on *in vivo* development, embryos were transferred into surrogate mothers 24 h after injection for offspring production. Although it is preliminary, we succeeded in the delivery of pups after ROSI with digitonin pretreatment (8 pups out of 14 transferred embryos). Thus, digitonin pretreatment is suggested to improve the success rate of ROSI.

Table 1. Fertilization and in vitro development after ROSI with digitonin

	6 h after ROSI[a]				72 h after ROSI	
Concentration of digitonin (μg/mL)	No. of oocytes with 1 Pb2	Female pronucleus + 1 PB2 Normal mPN	Small mPN	No mPN	No. of cultured embryos	Morula/ Blastocyst (%)
0	100	47 (47)[a]	5 (5)	48 (48)	30	23 (77)
1	76	48 (63)	4 (5)	24 (32)	47	43 (90)
10	95	60 (63)[b]	7 (7)	28 (29)	60	51 (85)

Abbreviations: mPN, male pronucleus; 1 PB2, one second polar body.
Chi-square comparisons [a] versus [b], $P < 0.05$.

319 USE OF PENTOXIFYLLINE AND HYALURONIC ACID FOR STALLION SPERM SEPARATION

G. Kutvolgyi[A,B], *T. Suh*[B], *E. Carnevale*[B], *and G. Seidel Jr*[B]

[A]Faculty of Animal Science, University of Kaposvar, 7400 Kaposvar, Hungary; [B]Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO 80521, USA. Email: gseidel@colostate.edu

In this study, we reduced the volume of separating media and the time of centrifugation to increase the yield of viable sperm for ICSI when few sperm are available. Hyaluronic acid (HA) was used successfully in combination with swim-up for separating bull spermatozoa (Shamsuddin and Rodriguez-Martinez 1994 Anim. Reprod. Sci. 36, 61–75). The purpose of this study was to compare the effectiveness of mini-Percoll and swim-up for low numbers of sperm treated or non-treated with HA or pentoxifylline (PX), a molecule that stimulates sperm motility (Gradil and Ball 2000 Theriogenology 54, 1041–1047). Poor-to-medium quality frozen semen (0.5-mL straws, 200 million cells/mL) from 3 stallions was used 3 times each. Two straws were thawed and mixed. Aliquots of 100 μL semen were allocated to 7 treatments. Three aliquots were incubated at 38°C for 20 min before Percoll-centrifugation (P-NT: non-treated; P-PX: 3.5 mM PX; or P-HA: 1 mg/mL HA, each in 0.25 mL chemically defined handling medium (HCDM)); one aliquot was centrifuged through Percoll without incubation (P-CON: control). Our mini-Percoll was a two-layer gradient (0.4 mL 90% and 0.5 mL 45% Percoll) in a 1.5-mL microcentrifuge tube that was centrifuged at 600*g* for 5 min. Then a 30-μL pellet was washed in HCDM at 300*g* for 5 min. At the same time, 3 aliquots of 100 μL sperm were placed in 1 mL HCDM for swim-up (S-NT: non-treated; S-PX: 3.5 mM PX; or S-HA: 1 mg/mL HA) for 30 min. A 0.65-mL aliquot of supernatant was centrifuged in HCDM at 300*g* for 5 min. In every treatment, the final 30-μL pellet aspirated from the bottom was evaluated. Recovery rate was determined using a hemacytometer. Sperm head, tail and acrosome membrane integrity were evaluated with Kovacs-Foote staining (Kovacs and Foote 1992 Biotech. Histochem. 67, 119–124) counting 300 sperm/sample. Cells were classified into 5 categories: intact head, tail and acrosome membrane (Intact); intact head, tail, damaged acrosome (IHITDA); intact head, damaged tail; damaged head, intact tail; damaged head, tail, acrosome. P-CON and P-PX resulted in more intact sperm compared to all swim-ups. IHITDA was nonsignificantly ($P > 0.05$) higher in P-PX than in P-CON (17 vs. 11%). Better recovery rates were found in P-CON, P-PX, and S-HA than in P-NT, P-HA, S-NT and S-PX. Hyaluronic acid increased recovery rate during swim-up, but not viability in any of the treatments. Pentoxifylline is beneficial if Percoll separation is delayed, but clarification of its effect on acrosome exocytosis is needed.

Table 1. Intact and recovery rates (LS means ± SE)

Treatment	P-CON	P-NT	P-HA	P-PX	S-NT	S-HA	S-PX
Intact (%)	54 ± 3.4^{a}	$51 \pm 3.4^{a,b}$	$50 \pm 3.4^{a,b,c}$	57 ± 3.4^{a}	$40 \pm 3.4^{b,c,d}$	$37 \pm 3.4^{c,d}$	36 ± 3.4^{d}
Recovery (%)	13 ± 1.4^{a}	5 ± 1.4^{b}	5 ± 1.4^{b}	13 ± 1.4^{a}	4 ± 1.4^{b}	12 ± 1.4^{a}	2 ± 1.4^{b}

[a,b,c,d] Means within rows without common superscripts differ ($P < 0.0001$).

320 USE OF SYNTHETIC HYALURONAN OR POLYVINYLPYRROLIDONE FOR INTRACYTOPLASMIC SPERM INJECTION INTO MOUSE OOCYTES

P.N. Moreira, *J. De la Fuente*, *A.T. Palasz*, *and A. Gutiérrez-Adán*

Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Madrid, Spain. Email: palasz@inia.es

The use of polyvinylpyrrolidane (PVP) in intracytoplasmic sperm injection (ICSI) seems to be exclusively related to its surfactant and colloidal properties. In contrast to PVP, which can be toxic to mouse embryos, hyaluronan (HA) is a biological compound. In addition to its colloidal

property, HA plays an important biochemical role in cell proliferation and migration and can be found intracellularly in the cleaving stage of mouse, sheep and primate embryos (Hunter RHF 1994 Mol. Reprod. Dev. 39, 176–181). We expect that the viscoelastic properties of HA in combination with its physiological functions may benefit the ICSI procedure. Oocytes at MII stage were collected from CD-1 mice 14 h after hCG injection (h-pi) and were kept at 37°C in KSOM medium for 30 min before ICSI. Semen used for injection was frozen by direct plunge into liquid nitrogen in M2 medium without cryoprotectants. Samples were thawed at 25°C in the air and mixed (1:5) with M2 medium containing either 10% PVP; 360000 MW (w/v; Sigma, St. Louis, MO, USA) or 60% (v/v) synthetic HA (s-HA; MAP-5; Bioniche Inc, Belleville, ON Canada) with comparable viscosity. Injections were performed at 25°C using a mercury-containing pipette attached to a piezo impact unit (Prime Tech, Ibaraki, Japan). A total of 239 oocytes (115 PVP and 124 s-HA) were injected in groups of ten in four replicates. Individual sperm heads decapitated by the freeze/thaw procedure were injected into oocytes and kept for 15 min at 25°C. Oocytes that survived ICSI were placed in 35 μL drops of KSOM medium (~15 zygotes per drop) under paraffin oil at 37°C and 5% CO_2 in humidified air. Cleavage and developmental rates were recorded at 24, 48, and 96 h after oocyte injection. Embryos which developed to the blastocyst stage were transferred to pseudo-pregnant females mated with vasectomized males. At Day 13, recipient mice were sacrificed and the number of implantations and fetuses were recorded. Data were compared between groups by Chi-square analysis. Significantly ($P < 0.05$) more embryos survived ICSI in PVP (74%) than in s-HA group (56%), which was primarily related to sperm adhesiveness to the injection pipette. However, there were no differences in developmental rates at any stage of *in vitro* embryo culture between groups (2 cell, 93 vs. 100%; 4–8 cell, 100 vs. 100%; blastocyst, 44 vs 50%) for PVP and s-HA, respectively. Significant differences ($P < 0.05$) between groups were observed in embryo implantation rates. When ICSI was performed with s-HA, 29 out of 35 blastocysts (83%) transferred to synchronized recipients were implanted, which was accomplished only by 19 of the 35 from the PVP group (54%). However, there was no difference between groups in the number of fetuses detected (8 (23%) vs. 9 (26%) for PVP and s-HA, respectively). The use of s-HA for mouse ICSI can be a valuable alternative to PVP. Hyaluronan may show further benefit if sperm adhesiveness to the micropipette can be eliminated, and may be superior to PVP if embryo implantation rates in the s-HA group can be sustained.

The authors would like to thank Bioniche, Inc., Belleville, ON, Canada for donating MAP-5.

321 DEVELOPMENT *IN VIVO* AND *IN VITRO* OF PORCINE OOCYTES FERTILIZED BY INTRACYTOPLASMIC INJECTION OF A FREEZE-DRIED SPERM HEAD

M. Nakai[A], *K. Kikuchi*[B], *A. Takizawa*[A], *M. Ozawa*[B], *J. Noguchi*[B], *H. Kaneko*[B], *M. Shino*[A], *and N. Kashiwazaki*[A]

[A]Graduate School, Azabu University, Kanegawa 229-8501, Japan; [B]National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan. Email: da0304@azabu-u.ac.jp

The present study investigated the development *in vivo* and *in vitro* of *in vitro* matured porcine oocytes injected with a freeze-dried (FD) boar sperm head. In mice, DNA damage was induced during the holding period after rehydration and before sperm injection (Wakayama, T. and Yanagimachi, R. 1998, Nat. Biotechnol., 16, 639–641). Here, we examined the relationship between duration of rehydration of FD sperm and *in vitro* development of FD sperm-injected porcine oocytes. We also assessed the *in vivo* developmental competence of the injected oocytes after embryo transfer. Ejaculated boar spermatozoa were suspended in Pig-FM (Suzuki, K. *et al*. 2002, Int. J. Androl. 25, 84–93) and sonicated for 1 min to separate sperm heads from the tails. An aliquot (100 μL) of the sperm suspension was put into a glass tube and then pre-cooled at −40°C for 6 h. Each tube was attached to a freeze-dry system (DuraDry μP, FTS Systems, Stone Ridge, NY, USA) for 12 h. The ampules were closed and stored at 4°C for more than 7 days before use. For rehydration, 100 μL of distilled water was added into the ampules. In Experiment I, we injected FD sperm heads which were kept for 0–60, 60–120, or 120–180 min after rehydration. At 1 h after the injection, the injected oocytes were stimulated with a DC pulse and cultured for 6 days. The rate of blastocyst formation and the number of cells in the blastocysts were examined. Embryos after *in vitro* fertilization (IVF) were evaluated as a control. As shown in Table 1, the rates of blastocyst formation were not different (by χ^2 test) for duration of rehydration and the control. However, the cell numbers of FD groups were lower ($P < 0.05$; by Student's *t*-test) than that in the control. In Experiment II, oocytes injected with a single FD sperm head and stimulated were transferred to both oviducts of a total of ten recipient gilts. Two recipients were diagnosed as pregnant at Day 30 of gestation. At Day 39, one of the pregnant recipients had an abortion, and two fetuses were recovered. The other pregnancy was not maintained. The results suggest that oocytes fertilized with a single FD sperm head have competence to be implanted and to develop to the early fetal stage, and also that the duration for rehydration does not influence *in vitro* developmental ability in pigs.

Table 1. Effects of the duration from rehydration of freeze-dried sperm heads to the injection of the heads into *in vitro* matured oocytes on *in vitro* development of the oocytes in pigs

Fertilization method	Duration from rehydration to injection (min)	Total number of oocytes examined	Blastocysts formed (%)	Average cell number in blastocysts
FD injected	0–60	108	23 (21.3)	39.1[a]
	60–120	93	18 (19.4)	35.9[a]
	120–180	94	14 (14.9)	36.6[a]
IVF		337	82 (24.3)	52.7[b]

[a,b] Values with different letters are significantly different ($P < 0.05$).

322 DIFFERENTIAL DEVELOPMENT OF RABBIT EMBRYOS FOLLOWING MICROINSEMINATION USING SPERM AND SPERMATIDS

N. Ogonuki[A], *K. Inoue*[A], *H. Miki*[A], *Y. Hirose*[C], *H. Okada*[C], *N. Shimozawa*[C], *S. Takeiri*[D], *H. Nagashima*[B], *T. Sankai*[C], *and A. Ogura*[A]

[A]RIKEN Bioresource Center, Tsukuba, Ibaraki, 305-0074, Japan; [B]Department of Life Science, School of Agriculture, Meiji University, Kawasaki, 214-8571, Japan; [C]Tsukuba Primate Center, National Institute of Infectious Diseases,Tsukuba, Ibaraki, Japan; [D]Kitayama Labes Co., Ltd., Ina City, 396-0021, Japan. Email: ogonuki@rtc.riken.jp

Microinsemination is a technique that delivers male germ cells directly into the ooplasm. The efficiency of fertilization and subsequent embryo development after microinsemination varies with species and the male germ cells used. This study examined the developmental ability of rabbit embryos *in vitro* and *in vivo* following microinsemination using haploid male germ cells at different stages. First, we injected rabbit spermatozoa, elongated spermatids, and round spermatids into mouse oocytes to assess their oocyte-activating capacity. Mouse oocytes are a good experimental model for assessing the oocyte-activating capacity of male germ cells from different species. The majority of mouse oocytes were activated irrespective of the stage of rabbit male germ cells injected (77, 61, and 73% for spermatozoa, elongated spermatids, and round spermatids, respectively). By contrast, these male germ cells activated homologous rabbit oocytes at rates of 100, 59, and 29%, respectively. After 120 h in culture, 69, 55, and 13% of these activated rabbit oocytes (pronuclear eggs) developed into blastocysts, respectively. The rate of embryo development into blastocysts following round spermatid injection was significantly improved when oocytes were activated by an electric pulse shortly before microinsemination. The total number of cells was counted in embryos that reached the morula/blastocyst stages in culture using nuclear-staining with propidium iodide. The average cell number of embryos derived from elongated (89 ± 41; mean ± SD) or round spermatid (98 ± 34) injection was significantly lower than that of control embryos (*in vivo* fertilization) (211 ± 44) ($P < 0.01$). After 24 h in culture, some four- to eight-cell-stage embryos were transferred into the oviducts of pseudopregnant females. Normal pups were born from embryos involving sperm (4 offspring/16 transfers; 25%) and elongated spermatid (3/26; 12%) injection, but none from those involving round spermatid injection (0/68). These findings indicate that rabbit male germ cells acquire the ability to activate oocytes and to support subsequent embryo development as they undergo spermiogenesis. Immaturity of the nuclear genome or difficulty in coordinating the behavior of the male and female chromosomes might compromise embryo development.

Superovulation

323 THE QUALITY OF PREOVULATORY FOLLICLES DURING FINAL MATURATION IN COWS STIMULATED WITH oFSH AND A DEFINED LH SURGE

O. Algriany, *P. Vos*, *H. Groenendaal*, *A. van Gastel*, *B. Colenbrander*, *and S.J. Dieleman*

Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. Email: o.algriany@vet.uu.nl

Multiple preovulatory follicles developing upon superovulation (SO) are heterogeneous in quality, which may be the consequence of follicular development deviating from that in untreated cyclic cows. Therefore, we investigated follicle performance in terms of estradiol (E), progesterone (P), and testosterone (T) concentrations in the fluid of stimulated preovulatory follicles (FF), in particular at onset of final maturation as initiated by the LH surge. Pre-synchronized HF cows ($n = 25$) were treated with oFSH (Ovagen; ICP, Auckland, New Zealand) for SO, and FF were collected 2 h pre-LH surge ($n = 9$ cows), and 6 h ($n = 8$) and 22 h C($n = 8$) post-LH. At Day 9 (estrus = Day 0), a norgestomet ear implant (Crestar; Intervet International BV, Boxmeer, The Netherlands) was inserted, and SO treatment was started at Day 10 using oFSH i.m. twice daily in decreasing doses during 4 days (total dose 17 mL). Prostaglandin (22.5 mg PG; Prosolvin, Intervet) was administered i.m. concomitant with the 5th dose of FSH. Ear implants were removed 50 h after PG and then GnRH (0.021 mg Receptal; Intervet) was administered i.m. inducing the LH surge 2 h later. Ovaries were collected by laparotomy at the time of GnRH, and 8 and 24 h later and all follicles sized >10 and <16 mm were aspirated to collect FF (pre-LH, $n = 79$; 6 h post-LH, $n = 78$; and 22 h post LH, $n = 78$ follicles). For comparison, E and P in FF from pre-LH groups that had been collected previously in 2 other experiments of our group were studied: (1) 86 FF collected by ultrasound-guided aspiration of follicles >8 mm from 23 cows at 30 h after PG, that is, preceding the LH surge, following treatment with 3000 IU eCG i.m. (Folligon; Intervet) on Day 10 and 15 mg PG on Day 12; (2) 12 FF of the dominant follicle from 12 untreated cyclic cows after ovariectomy 48 to 62 h after onset of luteolysis, that is, shortly before the natural LH surge. The concentrations (ng/mL FF) of E, P, and T were estimated by our validated RIAs. Data (mean ± SEM) were analyzed by ANOVA. The levels of E, P and T of the oFSH group were pre-LH: 399 ± 35, 49 ± 6 and 13 ± 2; 6 h post-LH: 194 ± 11, 202 ± 12, and 14 ± 1; and 22 h post-LH: 35 ± 2, 200 ± 23, and 7 ± 1, respectively. Although the change in E and P levels between the different time points after LH is in agreement with that reported for untreated cyclic cows (Dieleman *et al*. 1983 J. Endocrinol. 97, 31–42), the concentrations were lower. However, the most striking finding was the significantly lower E in pre-LH FF after oFSH compared to that after eCG (1302 ± 82) or of cyclic cows (1942 ± 200). The P levels in FF of the respective pre-LH groups were not significantly different. The much lower E level after oFSH is possibly due to the low or even absent LH bioactivity for oFSH in comparison to eCG. It could also indicate a lower developmental potential of oocytes following oFSH. However, it is known that SO with oFSH results in regular yields of transferable embryos similar to that after eCG. In view of the high variability of the E level in FF, it is concluded that selection of follicles for high E levels is a prerequisite when investigating oocyte development and maturation.