334 EFFECT OF CYTOPLASMIC INJECTION OF dsRNA ON TRANSIENT EXPRESSION OF THE EGFP GENE MICROINJECTED INTO RAT EMBRYOS

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RNA interference (RNAi) using double-stranded RNA (dsRNA) is a useful tool to inhibit posttranscriptional specific gene expression in mammalian cells. We investigated the effects of cytoplasmic injection of dsRNAs on transient gene expression in rat embryos when the enhanced green fluorescence protein (EGFP) gene was injected into pronuclei of zygotes. Wistar strain females were superovulated by injections of eCG and hCG and mated with males of the same strain. Pronuclear stage embryos were collected 29 h after hCG injection. The construction of the transgene, the EGFP gene controlled under the CMV-IE promoter, was dissolved at a concentration of 5 μg/mL in DNA injecting buffer (10 mM Tris-HCl, 0.1 mM EDTA), and it (3–5 pL) was microinjected into the pronuclei of embryos. After injection, embryos were cultured in KRB (Toyoda Y and Chang M C. 1974. J. Reprod. Fertil. 36, 9–22) at 37.0°C in 5% CO_2 and 95% humidified air until observation. In Experiment I, transient expression of the EGFP gene after microinjection into pronuclei of zygotes was examined. The EGFP expression in the embryos was observed at 6 h intervals until 48 h after the injection using fluorescence microscopy. In Experiment II, the inhibitory effect of dsRNAs targeting the EGFP mRNA was investigated. The dsRNAs for the EGFP were synthesized with T7 RNA polymerase from PCR product amplified with a pair of hybrid primers for the EGFP and T7 RNA polymerase priming site. Then the dsRNAs (1 μg/μL) solution (3–5 pL) was injected into the cytoplasm of the DNA-microinjected embryos 1 h after microinjection. As a control, the TE buffer solution (3–5 pL) was injected into the cytoplasm of the DNA-microinjected embryos. These embryos were cultured and then observed by fluorescence microscopy 48 h after DNA injection. Fluorescent intensity of the embryos was captured and analyzed with image analyzing software (Scion Image, Scion Corporation, Frederick, MD, USA), and all data were analyzed by χ^2 test. In Experiment I, the initiation of the fluorescent embryos was observed 24 h after DNA microinjection, and the proportion of fluorescent embryos in five replicates reached maximum (67%, 106/159) at 48 h after DNA injection. As shown in Table 1, there was a difference ($P < 0.05$) in the rate of fluorescent embryos between the dsRNA-injected group (18.4%, 33/179; range of 3.0–42.3%) and controls (58.8%, 77/131; range of 42.9–100%) in five replicates of Experiment II. The results of the present study suggest that injection of dsRNAs targeting the EGFP mRNA into the cytoplasm had an inhibitory effect on the transient expression of the EGFP gene microinjected into pronuclei of rat embryos. The inhibition system of the present study provides a powerful tool to study specific gene silencing, gene function, and development of early embryos.

Table 1. Effect of cytoplasmic injection of dsRNA on transient expression of the EGFP gene microinjected into rat embryos

Cytoplasmic injection	No. of ova used	% of surviving embryos	% of cleaving embryos	% of embryos with detected EGFP
Control	152	86.2	43.5	58.8
dsRNA	280	63.9*	36.4	18.4*

* Significant differences within each column ($P < 0.05$).

Ultrasonography

335 A PROCEDURE COMBINING ISTAT® ANALYSIS WITH OPU TO STUDY BOVINE FOLLICULAR ENVIRONMENTS

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Our previous work successfully analysed follicular fluid (45/61, 74% sampling success) from live cows using a portable clinical gas tension analyser (iSTAT Corp., Princeton, NJ). However, the oocyte recovery rate (7/24, 29%) from preovulatory follicles (POF) was disappointingly low, perhaps due to a need for the second puncture to aspirate the remaining follicular contents. This work describes a modified procedure that allowed a follicular fluid (FF) sample to be collected and stored in a gas-impermeable manner and the remaining follicular contents to be directly aspirated with or without follicular flushing from a single puncture. This modified dual port slip-luer hub, designed for use with a disposable needle ovum pick-up (OPU) system (PieMed, Netherlands), used a low negative pressure (30 mmHg), medium flow rate (25 mL/min) aspiration system with syringe flushing. FF samples for analysis were collected and stored in glass capillary tubes and the rest of the follicular contents/flushings were collected into tubes containing 10 mL collection medium and searched for oocytes. The hub's larger port, with a fitted Y connector, allows an initial flow of FF to be drawn into a capillary tube which has a polyvinylchloride (PVC) powder plugged end. When the PVC powder is permeated with FF it forms a seal effectively capping the tube. The remaining FF is diverted down the other leg of the Y connector using negative pressure. Flushing medium can be injected through the smaller port into the follicle while negative pressure is interrupted. A pilot trial examined gas tension, pH and ion concentrations from POF, dominant (D), and hormonally stimulated (S) follicles. Twenty synchronised Friesian cattle were scanned daily and follicles were tracked and mapped. The POF and D follicles were sampled on Day 20 or Day 10 respectively of the estrous cycle. The cows were then subjected to four weekly stimulations (used CIDR-B device inserted Day 2 and removed Day 5, 120 mg NIH-FSH-P1 administered twice daily on Day 5 and 6). Follicles were sampled and aspirated on Day 7. Successful FF analysis was achieved in 88% (110/125) of follicles attempted while oocyte recovery rates from

POF, D, and S follicles were 56% (9/16), 54% (6/11) and 43% (36/83) respectively, showing it is possible to combine portable gas tension analysis with effective OPU. However, further work is needed to elucidate the correlation between the follicular environment and the oocyte developmental competence.

Table 1. Follicular fluid sample analysis

Sample	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	pH	iCa (mM)	K (mM)	Na (mM)
S ($n = 83$)	84.4 ± 2.02	42 ± 0.47	7.46 ± 0.005	1.19 ± 0.01	$3.8 \pm 0.2^{***}$	143.9 ± 0.3
D ($n = 11$)	88.5 ± 4.11	38.5 ± 1.14	7.47 ± 0.018	1.2 ± 0.02	$3.8 \pm 0.4^{***}$	142.8 ± 0.6
POF ($n = 16$)	$75.7 \pm 3.12^{***}$	39.6 ± 0.67	7.48 ± 0.006	1.15 ± 0.02	3.5 ± 0.3	$145.5 \pm 0.4^{***}$
Arterial serum ($n = 9$)	101 ± 2.24	42.1 ± 1.24	$7.53 \pm 0.014^{***}$	1.18 ± 0.02	3.5 ± 0.5	141.9 ± 0.1

Within columns, *** $P < 0.001$ by ANOVA, Tukey's pairwise comparisons, values are means ± SEM.

336 COMPARISON OF FETAL DEVELOPMENT AND FETAL HEARTBEAT BY ULTRASONOGRAPHY IN ARTIFICIALLY INSEMINATED AND EMBRYO-TRANSFERRED DAIRY COWS

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Bovine fetal size and fetal heartbeat are important factors for detecting abnormal pregnancies in artificially inseminated and embryo-transferred dairy cows. The purpose of this study was to compare fetal growth and fetal heartbeat by transrectal ultrasonography in the early pregnancy of artificially inseminated (AI) and embryo-transferred (ET) dairy cows. The pregnancies of 86 animals were examined by fetal heartbeat count ($n = 35$ per animal of AI versus $n = 51$ per animal of ET) every 5 days from 35 to 60 days after breeding (the day of ovulation = day 0). The pregnancies of 237 animals were measured by fetal crown-rump length ($n = 95$ per animal of AI versus $n = 142$ per animal of ET) at 30 days and 60 days after breeding. Built-in electronic calipers were used to measure fetal dimensions on the ultrasonic screen. In addition, the times of first fetal heartbeats were recorded. Data were analyzed using ANOVA. The results are presented in the Table (Values were mean ± SD except for No. of animals). There were no differences in the range of fetal heartbeat for AI and ET (161.0 to 185.3 beats min^{-1} versus 169.7 to 184.6 beats min^{-1}). However, fetal heartbeats of AI at 35 days of gestation was higher ($P < 0.05$) than those in cows at days 40, 45, 50, 55 and 60 of gestation (185.3 ± 8.7 beats min^{-1} versus 161.0 ± 9.5, 165.0 ± 19.5, 170.3 ± 26.8, 169.4 ± 27.8, and 171.2 ± 11.9 beats min^{-1}, respectively). There were no differences in fetal crown-rump length between AI and ET. The prediction equation for crown-rump length in relation to fetal age of AI was $y = 0.0046x^2 - 0.2452x + 4.4424$, $R^2 = 0.9643$, while that for ET was $y = 0.0049x^2 - 0.2699x + 4.9396$, $R^2 = 0.9728$. Crown-rump length at 30 days and 60 days of gestation did not differ between AI and ET (1.2 ± 0.1 cm versus 1.2 ± 0.1 cm, 6.4 ± 0.3 cm versus 6.5 ± 0.4 cm, respectively). In conclusion, there were no differences in fetal development and heartbeats between AI and ET dairy cows. In addition, there were no differences in fetal crown-rump length between AI and ET dairy cows.

Table 1. Comparison of fetal development and heartbeat by ultrasonography in AI and ET dairy cows

Fetal age	Heart beats (beats min^{-1})				Crown-rump length (cm)			
	n	AI	*n*	ET	*n*	AI	*n*	ET
30	–	–	–	–	14	1.2 ± 0.1	21	1.2 ± 0.1
35	6	185.3 ± 8.7^{a}	5	169.7 ± 17.1	17	1.5 ± 0.2	22	1.5 ± 0.2
40	7	161.0 ± 9.5^{b}	8	171.4 ± 13.6	16	2.1 ± 0.1	20	2.1 ± 0.3
45	6	165.0 ± 19.5^{b}	9	183.2 ± 21.0	17	2.7 ± 0.3	21	2.6 ± 0.2
50	6	170.3 ± 26.8^{b}	9	176.9 ± 9.6	18	3.6 ± 0.4	20	3.6 ± 0.3
55	5	169.4 ± 27.8^{b}	7	173.0 ± 14.2	15	4.8 ± 0.6	20	4.8 ± 0.4
60	5	171.2 ± 11.9^{b}	9	184.6 ± 32.5	12	6.4 ± 0.3	18	6.5 ± 0.4

^{a,b} Unlike superscripts differ ($P < 0.05$).

337 OVUM PICK UP IN NON-PREGNANT AND POSTPARTUM SWAMP BUFFALOES (*BUBALUS BUBALIS*) AFTER FSH PRETREATMENT

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The feasibility of OPU has already been reported in prepubertal swamp buffaloes and heifers (Promdireg *et al*. 2000 Thai J. Vet. Med. 30(1), 41–50; Techakumphu *et al*. 2003 Theriogenology 61, 1705–1711) and it has to be emphasized that the reproductive potential of females can be maximized,

if OPU and IVEP are applied in the course of non productive periods such as anestrus and postpartum. The objective of this study was to evaluate the efficiency of Ovum Pick Up (OPU) in non lactating, pluriparous non-pregnant ($n = 5$) and lactating, postpartum swamp buffaloes, started 3 mts after calving ($n = 6$) with gonadotropin stimulation. OPU was performed every two weeks in the two groups of animals for a total of 6 sessions. To buffaloes receiving hormonal stimulation, a total of 400 mg of FSH was administered for three days in decreasing dose together with 100 μg of GnRH 24 h after the last FSH injection. The number of aspirated follicles between non-pregnant and postpartum buffaloes was not significantly different, 7.2 ± 3.7 (217) and 9.0 ± 3.2 (285), respectively ($P > 0.05$). Recovered oocytes between the two groups of hormonally stimulated animals was also not statistically different: 3.7 ± 2.7 (112) in the non pregnant and 5.9 ± 3.5 (198) in the postpartum group, respectively ($P > 0.05$). Among stimulated buffaloes, most aspirated follicles were of the small size (<5 mm). Overall oocyte recovery rate in both the groups was 61.8% (41–77.4%). Collectively the majority of recovered oocytes were single- and multi-layered, at a higher rate in non-pregnant than postpartum buffaloes. In conclusion, as already reported in cattle, this study confirms the possibility of retrieving oocytes by OPU from non-pregnant and postpartum buffaloes. The number and quality of recovered oocytes was similar in both groups of buffaloes.

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338 SYNCHRONISATION OF OVULATION IN MERINO EWES WITH GnRH IN THE BREEDING AND NON-BREEDING SEASON

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The objective was to determine the effect of GnRH as an aid to synchronise the time of ovulation in Merino ewes during the non-breeding and breeding seasons as determined by transrectal ultrasound. Oestrus was synchronized in 20 nulliparous Merino ewes (11–12 months old; 2 replicates of 10 animals) during spring 2003 and autumn 2004 at Camden, NSW, Australia, using FGA sponges for 12 days (30 mg Ovagest, Bioniche Pty. Ltd., Armidale, NSW) and an i.m. injection of 400 IU of PMSG (Pregnecol, Bioniche Pty. Ltd., Armidale, NSW). Ultrasound evaluations of ovaries were recorded on VHS tapes every 12 h for 36 h starting at sponge removal (SR), then half of the animals received an i.m. injection of 40 μg synthetic GnRH (Fertagyl, Intervet Australia Pty. Ltd, Bendigo, VIC) and ultrasound evaluations were conducted every 6 h until 60 h. The positions of the largest follicles were recorded on ovarian maps and their growth was monitored. Time of ovulation was defined as the time of disappearance of the largest follicle from the ovary. Ten days after ovulation, the position and diameter of the CL was confirmed by ultrasound. Comparisons were made between treated and control animals, and between breeding and non-breeding seasons, using *t*-tests. During the non-breeding season ovulation took place from 42 to 54 h (mean 48 ± 2.83 h) vs. 42 to 60 h (mean 52.2 ± 5.69 h) after SR in GnRH-treated vs. control animals ($P < 0.05$), respectively. Ovulation was delayed in the breeding compared with the non-breeding season ($P < 0.05$), starting from 48 to 60 h after SR for treated (52.8 ± 3.79 h) and control animals (57.0 ± 4.24 h; $P < 0.05$). These results suggest that GnRH synchronized the time of ovulation compared with the controls but the time of ovulation was later in the breeding than in the non-breeding season.

339 ULTRASOUND GUIDED OVUM PICK UP (OPU) IN PREPUBERTAL SWAMP BUFFALO USING THREE DIFFERENT VACUUM PRESSURES

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Our group successfully developed ovum pick-up in prepubertal swamp buffalo, however the quality of the oocytes that were collected was poor especially those without a cumulus mass (Techakumphu *et al*. 2003 Theriogenology 61, 1705–1711). The vacuum pressure used for oocyte collection was one of the factors influencing oocyte quality (Bols *et al*. 1997 Theriogenology 47, 1221–1236). The objective of this study was to compare the effect of three vacuum pressures on both the recovery rate and oocyte quality in prepubertal swamp buffaloes. Oocyte recovery and oocyte quality were using different groups of aspiration vacuum pressures. The maturation stages of the recovered oocytes were immediately assessed by fixation and rapid staining with basic carbol fuchsin. Twelve prepubertal calves, aged 1.5 yrs were a total of 180 mg FSH given, twice a day, in divided doses over 3 d (40/40, 30/30, 20/20). The animals were randomized into 3 groups, according to the different vacumm pressures, 100 ($n = 8$), 80 ($n = 8$) and 60 mmHg ($n = 8$). Two sets of treatments, carried out, with a 2 month interval between them. The oocyte recovery rates using 100 and 80 mmHg, were not different at 78.4% (29/37) and 83.6% (61/73). The 60 mmHg gave a lower rate, 65.7% (23/35) which was statistically different from the 80 mmHg group ($P < 0.05$). The oocytes recovered per donor showed no significant difference among the groups; 5.8 ± 4.9 for 100 mmHg, 7.6 ± 8.6 for 80 mmHg and 3.3 ± 2.1 for 60 mmHg respectively. The percentage of cumulus-oocyte complex (COC), single layered+partial cumulus oocytes (S + P) and expanded cumulus oocytes (EXP) showed no differences for any of the pressures, being 79.3, 65.5 and 82% of the total recovered. The experiment showed that follicles with a size of 2–6 mm were dominant after FSH treatment, being around 80% of the total number. Furthermore, the maturation stages of these oocytes were at prophase I and metaphase I. In conclusion, the vacuum pressure used for the oocyte retrieval technique influenced the recovery rate but not the oocyte quality.

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