ELSEVIER

Animal Reproduction Science 76 (2003) 155–161

ANIMAL REPRODUCTION SCIENCE

www.elsevier.com/locate/anireprosci

Collection of oocytes through transvaginal ultrasound-guided aspiration of follicles in an Indian breed of cattle

R.S. Manik*, S.K. Singla, P. Palta

Embryo Biotechnology Centre, National Dairy Research Institute, Karnal, Haryana 132 001, India

Received 6 June 2002; received in revised form 23 October 2002; accepted 8 November 2002

Abstract

The present study was undertaken in Karan Fries, an Indian breed of cattle to (1) determine the number of follicles available for puncture and (2) explore the potential of this breed as a donor of developmentally competent oocytes. Ovum pick-up (OPU) was performed using an ultrasound machine with a transvaginal convex transducer (5 MHz) with a needle guide, single lumen 19-gauge 60 cm long needle and a vacuum pressure of 90 mmHg. The number and size of follicles in each ovary was determined before puncture. The follicles were characterized on the basis of their diameter as small (3–5 mm), medium (6–9 mm) and large (≥10 mm). The oocytes recovered were classified by quality. They were matured in vitro, irrespective of their grade, in 50 μl droplets of the in vitro maturation (IVM) medium (TCM-199 + 10% fetal bovine serum (FBS) + 5 μg/ml follicle stimulating hormone (folltropin) + 1 μg/ml estradiol-17ß + 0.2 mM sodium pyruvate), covered with paraffin oil, in 35 mm petridish for 24 h in a CO_2 incubator (5% CO_2 in air) at 38.5 °C. The cleavage rate was recorded at day 2 post-insemination after subjecting the oocytes to in vitro fertilization (IVF). The differences in follicular populations of all size categories among individual donors were not significant. A total of 92 oocytes were recovered by aspiration of 157 follicles, with an overall recovery rate of 59% (range 35–79%). Of these, 32% were of grades A and B and the rest of grades C and D. The mean numbers of total follicles and the oocytes recovered per session did not differ significantly among individual donors. Out of the 73 oocytes subjected to IVM and IVF, 24 reached 2–4 cell stage at day 2 post-fertilization, with a cleavage rate of 33%. The total number of oocytes recovered was correlated with the number of small ($R = 0.54$, $P < 0.01$) but not with the number of medium and large follicles. This study demonstrates the use of OPU as a means of obtaining developmentally competent oocytes from an Indian breed of cattle for obtaining cattle oocytes in India where cow slaughter is not allowed for religious reasons.

Keywords: Aspiration; Cattle-reproductive technology; Follicle; IVF; Oocyte; Ovum pick-up

* Corresponding author. Fax: +91-184250042.
E-mail address: manik_rs@rediffmail.com (R.S. Manik).

0378-4320/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0378-4320(02)00241-5

1. Introduction

Development and application of reproductive technologies like in vitro embryo production through in vitro maturation, fertilization and culture (IVMFC) of oocytes, production of cloned or transgenic cattle, establishment of oocyte banks, etc. can be expected to bring about a significant increase in the population of superior genetic merit cattle. However, cattle oocytes, which are a basic requirement for development of these technologies, are not available in India due to the ban on cow slaughter for religious reasons. Ovum pick-up (OPU) from live animals appears to be the only means available for obtaining cattle oocytes on a large scale. The oocyte yield in OPU depends upon the number of follicles available for puncture, which are influenced, besides other factors, by breed, nutritional status and climatic conditions. The potential of Indian breeds of cattle to act as oocyte donors can, therefore, be expected to differ from that of exotic breeds. Moreover, cattle reared in India are subjected to nutritional stress since they are fed mainly roughages and crop residues. They are also subjected to heat stress because of the tropical climate marked by high temperature and humidity. Both these types of stress have significant adverse effects on follicular dynamics (Draincourt, 2001; Boland et al., 2001).

Although collection of oocytes through OPU is routinely done in the *Bos taurus* breed of cattle, there is no information available in any Indian breed of cattle on the number of follicles available for puncture or the oocyte yields possible. The present study was, therefore, undertaken in Karan Fries, an Indian breed of cattle to (1) determine the number of follicles available for puncture and (2) explore the potential of this breed as a donor of developmentally competent oocytes. Karan Fries is an Indian breed of cattle that is popular because of its high milk production capability. It has been developed by crossing zebu with Holstein Friesian breed. Preliminary work from this study has been reported earlier as an abstract (Manik et al., 2002b).

2. Materials and methods

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Folltropin was obtained from Vetrepharm Canada Inc., Bellville, ON, Canada. Disposable petridishes were from Becton, Dickinson and Co. (Lincoln Park, NJ, USA), and the 0.22 and 0.45 μm filters were from Millipore Corp. (Bedford, MA, USA). Mineral oil was from Squibb and Sons (Princeton, NJ, USA).

2.1. Oocyte aspiration from live cows

Non-lactating acyclic Karan Fries cattle ($n = 4$), between 10 and 13 years of age, which had not been subjected to follicular aspiration, previously were chosen for the study. An epidural anaesthetic of 3–4 ml lignocaine hydrochloride (2%) was given immediately before follicle aspiration for ease of manipulation. Follicular aspiration was performed using an ultrasound machine (Aloka SSD-500) with a transvaginal convex transducer (5 MHz) with a needle guide, single lumen 19-gauge 60 cm long sterile needle with an ultrasound echo tip (Cook Veterinary Products, Qld, Australia) and a vacuum pressure of 90 mmHg as described

earlier (Manik et al., 2002a). The number and size of follicles in each ovary was determined before puncture. The follicles were characterized on the basis of their diameter as small (3–5 mm), medium (6–9 mm) and large (≥10 mm). All the visible follicles were punctured. The flushing medium was Dulbecco's phosphate buffered saline (DPBS) supplemented with 50 μg/ml gentamycin, 20 μg/ml heparin and 0.3% lyophilized bovine serum albumin (BSA).

2.2. In vitro maturation of oocytes

The oocytes recovered were transferred to the holding medium which consisted of tissue culture medium (TCM)-199 + 10% fetal bovine serum (FBS). They were classified as grade A cumulus-oocyte complexes (COCs) with an unexpanded cumulus mass having at least five layers of cumulus cells, and with homogenous cytoplasm; grade B COCs with 2–4 layers of cumulus cells, and with homogenous cytoplasm; grade C oocytes partially denuded of cumulus cells and/or with irregular shrunken cytoplasm, and grade D oocytes completely denuded of cumulus cells and/or with irregular shrunken cytoplasm (Chauhan et al., 1998). The oocytes were washed three times with the in vitro maturation (IVM) medium that consisted of TCM-199 supplemented with 10% FBS, 5 μg/ml follicle stimulating hormone (Folltropin), 1 μg/ml estradiol-17ß and 0.2 mM sodium pyruvate. The recovered oocytes, irrespective of their grade were matured in 50 μl droplets of the IVM medium, covered with paraffin oil, in 35 mm petridish for 24 h in a CO_2 incubator (5% CO_2 in air) at 38.5 °C.

2.3. In vitro fertilization of oocytes

The spermatozoa were prepared for insemination by modification of a method described earlier (Suzuki et al., 1992). Briefly, the contents of two 0.25 ml straws of frozen cattle semen were thawed in water at 35–37 °C. The semen washed twice with Brackett and Oliphant (BO, Brackett and Oliphant, 1975) medium (without BSA and caffeine), containing 10 μg/ml heparin by centrifugation at 1800 rpm for 5 min each. The spermatozoa were suspended in BO medium containing 0.5% fatty acid-free BSA, 10 μg/ml heparin and 5 mM caffeine, and 100 μl droplets of these were incubated for 1 h at 38.5 °C in a CO_2 incubator (5% CO_2 in air) before insemination of in vitro cultured oocytes. After 24 h of in vitro culture, the oocytes were washed in BO medium (containing fatty acid-free BSA, heparin and caffeine) and introduced into 100 μl droplets of processed spermatozoa (10–12 million spermatozoa/ml) and were left for 6 h in a CO_2 incubator (5% CO_2 in air) at 38.5 °C. At the end of sperm–oocyte incubation, the oocytes were separated from sperm droplets and washed with TCM-199 supplemented with 10% FBS and then cultured for 2 days. The cleavage rate was recorded on day 2 (42–44 h) of culture.

2.4. Statistical analyses

Correlations were done between the total number of oocytes recovered and the number of small, medium and large follicles. Follicles of various size categories and the number of oocytes recovered were compared between different animals by ANOVA after root transformation of data.

Table 1
Mean (±S.E.M.) number of small (3–5 mm), medium (6–9 mm) and large (≥10 mm) follicles during various sessions in individual donors

Animal no.	Puncture session (*n*)	Follicle size		
		Small	Medium	Large
1	9	6.9 ± 0.5	1.2 ± 0.6	1.4 ± 0.3
2	6	5.2 ± 0.8	0.5 ± 0.3	0.5 ± 0.2
3	4	3.7 ± 1.2	0.2 ± 0.2	1.0 ± 0.3
4	4	2.7 ± 1.0	0.2 ± 0.2	0.5 ± 0.2
Total	23	5.2 ± 0.5	0.7 ± 0.1	1.0 ± 0.2

3. Results

The mean number of follicles of various size categories among individual donors is shown in Table 1. The differences in follicular populations of all size categories among individual donors were not significant. A total of 92 oocytes were recovered by aspiration of 157 follicles, with an overall recovery rate of 59% (range 35–79%, Table 2). The mean numbers of total follicles and the oocytes recovered did not differ significantly among donors. The number of oocytes of various grades recovered from individual donors and the post-fertilization cleavage rate are given in Table 3. Of the 92 oocytes recovered, 32% were

Table 2
Mean (±S.E.M.) number of follicles punctured, oocytes recovered and the recovery rate during various sessions in individual donors

Animal no.	Puncture session (*n*)	Punctured follicles		Oocytes recovered		Recovery rate (%)
		n	Mean ± S.E.M. (range)	*n*	Mean ± S.E.M. (range)	
1	9	86	9.5 ± 0.9 (6–12)	53	5.9 ± 0.8 (1–10)	62
2	6	37	6.2 ± 0.9 (3–10)	21	3.5 ± 1.4 (1–7)	57
3	4	20	5.0 ± 1.5 (2–10)	7	1.7 ± 0.4 (1–3)	35
4	4	14	3.5 ± 0.7 (2–6)	11	2.7 ± 0.4 (2–4)	79
Total	23	157	6.8 ± 0.7	92	4.0 ± 0.5	59

Table 3
Number of oocytes of various grades recovered and the post-fertilization cleavage rate in individual donors

Animal no.	Quality of oocytes recovered				Oocytes cultured	Oocytes cleaved	Cleavage rate (%)
	A grade *n* (%)	B grade *n* (%)	C grade *n* (%)	D grade *n* (%)			
1	4 (7)	14 (26)	15 (28)	20 (38)	37	20	54
2	3 (14)	5 (24)	9 (43)	4 (19)	19	0	0
3	0 (0)	0 (0)	5 (71)	2 (28)	7	2	29
4	0 (0)	3 (27)	3 (27)	5 (45)	10	2	20
Total	7 (8)	22 (24)	32 (35)	31 (34))	73	24	33

of grades A and B and the rest of grades C and D. Out of the total 73 oocytes cultured and fertilized, 24 reached 2–4 cells stage at day 2 post-fertilization, with a cleavage rate of 33%. None of the oocytes recovered from Animal no. 3 exhibited cleavages in any of the sessions. The total number of oocytes recovered was correlated with the number of small ($R = 0.54$, $P < 0.01$) but not with the number of medium and large follicles.

4. Discussion

To our knowledge, this is the first report on collection of oocytes from live cattle and their IVMFC from any Indian breed. Since cow slaughter is banned in India, lack of availability of cattle oocytes is the major factor limiting the development of reproductive technologies suitable for Indian cattle.

In the present study, the total number of follicles available for puncture and the number of oocytes recovered per animal per session was 6.8 ± 0.7 and 4.0 ± 0.5, respectively. These figures are lower than the corresponding values of 12.4 ± 6.1 and 5.4 ± 3.7, respectively, in Holstein heifers (Garcia and Salaheddine, 1998) and 14.7 ± 2.27 and 5.6 ± 1.18, respectively, in Simmental heifers (Goodhand et al., 1999). This highlights the influence of breed differences, environment and nutrition on the follicular populations of various size categories and the resultant oocyte yields possible. The potential of Karan Fries cattle to act as oocyte donors is, therefore, lower than that of exotic breeds of cattle.

This study also highlights the differences among individual animals in terms of their potential as donors of developmentally competent oocytes. Not only the mean number of oocytes recovered varied from 1.7 to 5.9 among individual donors, the post-fertilization cleavage rate also varied markedly from 0 to 54% among individual donors. The oocytes collected from Animal no. 2 did not exhibit cleavages in any of the sessions. Other authors have also observed such large variations in the developmental competence of the oocytes collected from live cattle through OPU (Hasler et al., 1995; Twagiramungu et al., 1999). The reasons for this variability are little understood. Nevertheless, it highlights the necessity to screen the donors prior to their selection. For developing commercial OPU–IVF programs, the donors need to be evaluated not only in terms of their potential as oocyte donors but also for the ability of their oocytes to exhibit post-fertilization cleavage and subsequent development up to blastocyst stage for transfer to recipients.

Since the operator has been found to significantly influence the recovery rate in earlier studies (Fry et al., 1997), the same operator performed OPU throughout the present study. The combination of 19-gauge needle and 90 mmHg vacuum pressure employed in our study was based upon the combination applied most frequently in cattle, i.e. 16–19-gauge needles and 75–100 mmHg vacuum pressure (Looney et al., 1994; Gibbons et al., 1995). The recovery rate of 59% in the present study is comparable to that reported earlier by other authors (Pieterse et al., 1991; Fry et al., 1997). The proportion of grade A + B/total number of oocytes which was 32% in the present study is lower compared to the earlier reports of 47 (Ward et al., 2000), 72 (Donnay et al., 1997) and 82% (Hasler et al., 1995). The needle gauge and the vacuum pressure have been reported to affect the quality of oocytes, with excessive pressure adversely affecting the oocyte viability, presumably due to stripping away the cumulus layers from around the oocyte (Fry et al., 1997). In a recent study, vacuum

pressure above 50 mmHg has been reported to adversely affect the oocyte quality (Ward et al., 2000). The vacuum pressure of 90 mmHg used in the present study could be high enough to cause stripping away of cumulus cells resulting in erroneous classification of some of the oocytes. Although the overall cleavage rate of 33% obtained in the present study is low, it could be because of not selecting the oocytes prior to IVMFC. Cleavage rates of grades C and D oocytes have been reported to be substantially lower than those for grades A and B oocytes (Ward et al., 2000).

The total numbers of oocytes recovered were correlated with the number of small but not with the number of medium and large follicles. This suggests that a major portion of oocytes were probably recovered from small follicles. In earlier studies also an inverse relationship has been obtained between follicle size and recovery rate, possibly because the larger follicles had the tendency to fold around the needle tip due to suction power, leaving the oocyte behind these folds, when follicular fluid was aspirated (Pieterse et al., 1991).

In conclusion, the present study demonstrates the use of OPU as a means of obtaining developmentally competent oocytes from an Indian breed of cattle. Since cow slaughter is not allowed in India for religious reasons, availability of oocytes through OPU can help provide developmentally competent oocytes on the laboratory bench for use in various reproductive technologies.

References

Boland, M.P., Lonergan, P., O'Callaghan, D., 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology 55, 1323–1340.

Brackett, B.G., Oliphant, G., 1975. Capacitation of rabbit spermatozoa in vitro. Biol. Reprod. 12, 260–274.

Chauhan, M.S., Singla, S.K., Palta, P., Manik, R.S., Madan, M.L., 1998. In vitro maturation and fertilization, and subsequent development of buffalo (*Bubalus bubalis*) embryos: effects of oocyte quality and type of serum. Reprod. Fertil. Dev. 10, 173–177.

Draincourt, M.A., 2001. Regulation of ovarian follicular dynamics in farm animals: implications for manipulation of reproduction. Theriogenology 55, 1211–1239.

Donnay, I., De Roover, R., van Langendonckt, A., Massip, A., Dessy, F., 1997. Overall efficiency of an experimental ovum pick-up program in cattle. Theriogenology 47, 155 (abstract).

Fry, R.C., Niall, E.M., Simpson, T.L., Squires, T.J., Reynolds, J., 1997. The collection of oocytes from bovine ovaries. Theriogenology 47, 977–987.

Garcia, A., Salaheddine, M., 1998. Effects of repeated ultrasound-guided transvaginal follicular aspiration on bovine oocyte recovery and subsequent follicular development. Theriogenology 50, 575–585.

Gibbons, J.R., Krisher, P.L., Carlin, S.K., Pearson, R.E., Gwazdauskas, F.C., 1995. In vitro embryo production after microinjection and ovarian dynamics following transvaginal follicular oocyte aspiration. Theriogenology 43, 1129–1139.

Goodhand, K.L., Watt, R.G., Staines, M.E., Hutchinson, J.S.M., Broadbent, P.J., 1999. In vivo oocyte recovery and in vitro embryo production from bovine donors aspirated at different frequencies or following FSH treatment. Theriogenology 51, 951–961.

Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., Trimmer, S.A., 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 43, 141–152.

Looney, S.R., Lindsey, B.R., Gonseth, C.I., Johnson, D.L., 1994. Commercial aspects of oocyte retrieval and in vitro fertilization (IVF) for embryo production in problem cows. Theriogenology 41, 67–72.

Manik, R.S., Chauhan, M.S., Singla, S.K., Palta, P., 2002a. Transvaginal ultrasound-guided aspiration of follicles from Indian buffaloes (*Bubalus bubalis*) with reproductive problems. Vet. Rec. 150, 22–24.

Manik, R.S., Singla, S.K., Palta, P., 2002b. Recovery and cleavage rate of oocytes retrieved through transvaginal ultrasound-guided aspiration of follicles in Indian cattle. Theriogenology 57, 791 (abstract).

Pieterse, M.C., vos, P.L.A.M., Kruip, T.A.M., Willemse, A.H., Taverne, M.A.M., 1991. Characteristics of bovine estrous cycles during repeated transvaginal ultrasound-guided puncturing of follicles for ovum pick-up. Theriogenology 35, 401–412.

Suzuki, T., Singla, S.K., Sujata, J., Madan, M.L., 1992. In vitro fertilization of water buffalo follicular oocytes and their ability to cleave in vitro. Theriogenology 38, 1187–1194.

Twagiramungu, H., Morin, N., Brisson, C., Carbonneau, G., Durocher, J., Bousquet, D., 1999. Animal factors that influence the in vitro production of bovine embryos. Theriogenology 51, 334 (abstract).

Ward, F.A., Lonergan, P., Enright, B.P., Boland, M.P., 2000. Factors affecting recovery and quality of oocytes for bovine embryo production in vitro using ovum pick-up technology. Theriogenology 53, 433–446.