Asian-Aust. J. Anim. Sci.
Vol. 20, No. 6 : 842 - 849
June 2007

www.ajas.info

Production of Kids from *In vitro* Fertilized Goat Embryos and Their Parentage Assessment Using Microsatellite Markers

D. Malakar[1, *], S. K. Das[1], M. Mukesh[2], M. Sodhi[2] and S. L. Goswami[1]

[1]Animal Biotechnology Center, National Dairy Research Institute, Karnal-132001, Haryana, India

ABSTRACT : The purpose of the present study was to produce live offspring from *in vitro* fertilized goat embryos. Oocytes were collected from abattoir ovaries and kept in oocyte collection medium. Oocytes were washed 4-5 times with maturation medium containing medium-199 with 5 μg/ml FSH, 100 μg/ml LH, 1 μg/ml estradiol-17β, 50 μg/ml gentamycin, 10% inactivated estrus goat serum, and 3% BSA (fatty acid free). Oocytes were placed in 100 μl drops of maturation medium containing granulosa cell monolayer and incubated in a 5% CO_2 incubator at 38.5°C for 27 h. For capacitation of spermatozoa fresh semen was processed and mixed in 3 ml fertilization TALP medium containing 50 μg/ml heparin and kept in the above incubator for 2 h. The capacitated spermatozoa were co-incubated with matured oocytes for fertilization. Cleaved embryos were separated and cultured in embryo development medium with oviductal cells and 494 embryos were produced. Recipient goats were synchronized with two injections of 15 mg $PGF_{2\alpha}$ /goat 10 days apart. Eighty early stage embryos were transferred into the uterotubal junction of 14 surrogate mothers using laparoscopy techniques. One recipient delivered twin kids, whereas another two recipients each delivered a single kid The parentage of these kids was evaluated using highly polymorphic co-dominant microsatellites markers. From the present study, it was concluded that live goat kids can be produced from *in vitro* matured and fertilized goat embryos, to the best of our knowledge for the first time in India. (**Key Words :** Goat, IVF, Kids, Laparoscopy, Microsatellite, Paternity)

INTRODUCTION

The biotechniques of *in vitro* maturation and fertilization of goat oocytes have immense potential for production of large number of embryos and their transfer to recipients for production of normal offspring and faster multiplication of superior germplasm (Keskintepe et al., 1994; Cognie et al., 2003). Slaughterhouse derived ovaries provide a cheap and abundant source of large number of oocytes for production of live goat kids (Martino et al., 1995; Cognie et al., 2004). Applications of this IVF technique include development of emerging biotechniques such as embryo sexing (Huang et al., 2001; O'Brien et al., 2004), nuclear cloning (Oppenheim et al., 2000; Tecirlioglu et al., 2003; Cowan et al., 2005) and transgenesis (Huang et al., 2001; Baldassarre et al., 2004).

In vitro production of embryos involves oocyte recovery, maturation, and fertilization with capacitated spermatozoa and culture of the resulting embryos. Maturation of oocytes is carried out in culture medium generally containing mediun-199 with FSH, LH, estradiol-17β, BSA, estrus goat serum etc. (Cognie et al., 2003). Different concentration of heparin, ranging from 2 to 100 μg/ml, and different media has been used for capacitation of goat fresh and frozen spermatozoa (Katska et al., 2004). Production of goat embryos and live kids from *in vitro* maturation and fertilization procedures has recently been improved using different macromolecule supplementation in medium (Herrick et al., 2004) and co-culture systems (Katska et al., 2004). First goat kid was born using the IVF techniques on ovulated oocytes in goat by Hanada (1985). Younis et al. (1991) was reported pregnancy in goats by embryo transfer after IVF of goat oocytes. Crozet et al. (1993) produced first goat kid from *in vitro* maturation and fertilization of goat oocytes.

Recipient goats were synchronized with $PGF2_{\alpha}$, gonadotropin releasing hormone and an ear implant of progestogen resulting in observed oestrus in 100, 67 and 50% animals respectively (Ruth-Macedo et al., 1999). It has

* Corresponding Author: D. Malakar. Tel: +91-0184-2259304, Fax: +91-0184-2250042, E-mail: dhrubamalakar@yahoo.com
[2] National Bureau of Animal Genetic Resources, PO Box 129, G. T. Road, Karnal-132001, Haryana, India.
Received April 22, 2006; Accepted August 23, 2006

been reported that 2 i.m. injections of $PGF2_{\alpha}$ (Estrumate) 12 days apart lead to oestrus expression observed significantly 67±5 h after treatment (Tamboura et al., 1997). Estrus could also be synchronized in recipient goats by using an intravaginal progesterone device CIDR® for 13 days and injection of 400 IU PMSG 48 h before CIDR® removal (Shin et al., 2004).

The major problems of embryo transfer through surgery in goats that the process is highly invasive and some time lead to post-operative adhesions of reproductive organs of goat (Hunter et al., 1995). These procedures also impair the subsequent fertility of goats. To overcome these disadvantages a quick, efficient and reliable laparoscopic technique has been reported for transfer of IVF embryos into uterine horn (Baldassarre et al., 2002) and fallopian tubes (Katska et al., 2004; Shin et al., 2004).

Microsatellites has become the marker of choice in many livestock species for paternity inference. Microsatellites or simple tandem repeats (STRs) are highly polymorphic genetic markers with co dominantly inherited alleles are relatively easy to score. They consist of strings of tandem repeats of short motifs ranging from mono to penta nucleotides with variability in the number of repeats (Litt and Luty, 1989; Fan et al., 2005; Sukla et al., 2006). Highly variable STR loci are common in mammalian genomes and can readily be typed by Polymerase Chain Reaction followed by electrophoresis on denaturing urea-polyacrylamide gel (Sodhi et al., 2006). These attributes make microsatellites the ideal marker to reveal parentage/genetic relationships between individuals. The objectives of the present study were to produce in *vitro* matured and fertilized goat embryos in Indian goat and their transfer to synchronized recipient animal using laparoscopy technique for production of normal live offspring to best of our knowledge first time in India. The parentage status of the IVF kids was confirmed by nine microsatellite marker based genotyping.

MATERIALS AND METHODS

All plastic wares were purchased from Tarson Products Co. (Kolkata, India) and chemicals/biochemicals from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise.

Collection of the oocytes from goat ovaries

Oocytes were isolated from goat ovaries collected slaughterhouse and categories into A grade (>5 layer COC), B grade (3-5 layer COC), C grade (<3 layer COC) and D (without layer COC). A grade & B grade oocytes were matured on the granulosa cell monolayer (Malakar and Majumdar, 2002) in maturation medium and kept in 5% CO_2 incubator at 38.5°C for 27 h. This medium contained medium-199 with 5 μg/ml FSH, 100 μg/ml LH, 1 μg/ml estradiol-17β, 50 μg/ml gentamycin, 10% inactivated estrus goat serum, and 3% BSA (fatty acid free).

In vitro fertilization

Fresh semen was collected from two bucks with artificial vagina in a single tube and mixed together. Fifty μl semen was added into 5 ml quenched sperm-TALP medium (Reed et al., 1996) and centrifuged at 320 g for 5 min. The pellet was dissolved in 5 ml fresh sperm-TALP medium and centrifuged again as above. Finally 2 ml of fertilization-TALP medium containing 50 μg/ml heparin (Katska et al., 2004) and kept in 5% CO_2 incubator at 38.5°C for 2 h. Capacitated spermatozoa were taken in 100 μl drops at the concentration of 2×10^6 spermatozoa/ml in Petri dishes and covered with mineral oil. Then 10-15 matured oocytes were added to each drop and kept in 5% CO_2 incubator at 38.5°C for 12 h.

In vitro embryo culture

After 12 h of co-incubation, fertilized oocytes were washed 4 to 5 times with embryo development medium (EDM) (Malakar and Majumdar, 2005) containing medium-199 with 100 μg/ml L-glutamine, 27 μg/ml sodium pyruvate, 50 μg/ml gentamycin, 3% BSA (fatty acid free), 5% FCS. The washed fertilized oocytes were further cultured in EDM with the oviductal epithelial cells (Izquierdo et al., 1999; Katska et al., 2002) and kept in 5% CO_2 incubator. Cleaved embryos were separated after 36 h and co-cultured in fresh EDM with oviductal epithelial cells (Izquierdo et al., 1999; Katska et al., 2002) and kept in 5% CO_2 incubator at 38.5°C.

Synchronization of recipient goats and transfer of IVF embryos

Recipient goats were synchronized with two injection of $PGF2_{\alpha}$ (Ruth-Macedo et al., 1999) Lutalyse® (Pharmacia, Belgium), 15 mg/goat, i.m. 10 days apart. Estrus symptoms were observed after 48 h of last injection. Recipient goats were kept overnight off-feed before transfer of IVF embryos on day 2 after estrous symptom. Embryos were loaded into 0.25 ml French straw and kept in 5% CO_2 incubator at 38.5°C before transfer. The recipient goat was injected Triflupromazine HCL (Siquil®, Sarabhai Zydus) at 0.1 mg/kg body weight i.v. and animal held in dorsal recumbency in head down position at an angle of 40° to the horizontal. The field of operation cranial to the mammary gland was washed and shaved followed by 5 ml/goat Lignocaine HCL (Xylocaine® 2%, AstraZeneca), was injected subcutaneous 10 cm cranial to the mammary gland in the left side of ventral midline. The trocar of endosecope was inserted at this injection point and observed the uterine

Table 1. Production of *in vitro* fertilized embryos from *in vitro* matured oocytes

Ovaries	Grade-A oocytes	Grade-B oocytes	Grade-C oocytes	Grade-D oocytes	A+B oocytes	Total oocytes	Total Embryos
1,977	1,092	1,174	1,350	1,596	2,266	5,212	494
	20.9%	22.5%	25.9 %	30.6%	43.4%	2.63	21.8%

Table 2. IVF goat embryos transfer and live kids born from surrogate mother

Recipient goats	Number of IVF embryos transferred	Number of pregnancies developed	Live kids born
14	80	3	4

horns. Then trocar for the grasping forceps was inserted about 15 cm cranial to the mammary gland right side of ventral mid line and uterine horn was taken out. Then embryos from French straw were transferred to uterotubal junction (Baldassarre et al., 2002) of recipient goats (2 to 5 embryos in each horn) through very simple laparoscopic (Karl Storz Endoskopie, Germany) technique. Incisions were sutured with catgut and nylon thread respectively and monitoring the recipient goats of postoperative care.

Microsatellite genotyping for parentage assessment

Blood samples were collected from each candidate parents and IVF offspring by jugular vein puncture in EDTA coated vacuutainer tubes. Genomic DNA from whole blood was extracted using standard phenol chloroform procedure of Sambrook et al. (1989). For assigning the paternity following a likelihood-based approach. DNA samples of candidate parents and offspring were genotyped using microsatellites markers (di-nucleotide repeats). Out of fifteen markers viz: BM1314, BM6506, BM6526, ILSTS029, ILSTS034, ILSTS044, ILSTS049, ILSTS059, ILSTS08, OarFCB304, OarJMP8, OarCP34, OarCP38, OarAE129, OarJMP29 analyzed, the six markers namely ILSTS08, ILSTS029, ILSTS034, OarFCB304, OarJMP8, OarJMP29, were not used in the final analysis being monomorphic. Primer sequences and chromosomal location of the microsatellite markers used in the present study are summarized in Table 3.

The primer sets for PCR amplification of microsatellite loci were synthesized from Sigma Aldrich, USA. PCR was carried out in 25 μl reaction volume containing 50-100 ng of template DNA, 50 ng of each primer, 200 μM of each dNTP (Promega Corporation, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.5 units of Taq DNA Polymerase (Promega Corporation, Madison, WI, USA). PCR reaction was carried out in PTC-100 thermocycler (MJ Research Inc., MA, USA). A Common "Touchdown" PCR programme used for amplification of all the 10 markers involved 3 cycles of 45 sec at 95°C, 1 min at 60°C; 3 cycles of 45 sec at 95°C, 1 min at 57°C; 3 cycles of 45 sec at 95°C, 1 min at 54°C; 3 cycles of 45 sec at 95°C, 1 min at 51°C and 20 cycles of 45 sec at 92°C, 1 min at 48°C (FAO, 1996).

The PCR products were initially analyzed on ethidium bromide (1.5 μg/ml) stained 2% agarose gel. The denatured PCR products were electrophoresed on prewarmed 6% Urea-polyacrylamide denaturing sequencing gel (30×38 cm) using Biorad Sequi: Gen GT apparatus at 75 W. The resolved alleles were visualized by silver staining procedure of Bassam et al. (1991). Allelic size range was estimated using 10 bp sequencing ladder (Invitrogen). Genotype of individual animal at each microsatellite loci was recorded by direct counting.

CERVUS 2.0 a Windows® based computer programme (Marshall et al., 1998) commonly used for parentage analysis using co-dominant loci was employed in the present study to carry out likelihood based analysis for determining the correct parentage of IVF offspring. The analysis involved comparison of candidate parent's genotypes against the offspring's genotype. Both exclusion and maximum likelihood analysis were conducted to exclude or to identify the most likely parent. The allele frequency base data was generated for each locus using the genotype profile to calculate the parentage parameters. The LOD score (log of the product of likelihood ratios at each locus) value for each candidate parent based on the genotypes of the candidate parents and offspring was obtained. The observed allelic frequencies data was simulated for 10,000 cycles to obtain the critical values of log-likelihood statistic delta for evaluating confidence and reliability in assigning parentage to the most likely candidate. Parentage was evaluated with 95% (strict) and 80% (relaxed) levels of stringency. Additionally, several standard genetic parameters viz., observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), null allele frequency (based on heterozygote deficiency) and an average exclusion probabilities at each locus were calculated to check the suitability of microsatellite markers used in the present study.

Humane animal cares and use

Permission was taken from institute ethics committee and their guidelines were followed during the present animal experiment.

RESULTS

In the present study, 5212 (2.63 per ovary) oocytes were isolated from 1977 goat ovary samples collected from

Figure 1. First IVF goat male kid with surrogate mother.

Figure 2. IVF goat kids with their surrogate mothers.

slaughterhouse. The oocytes were graded as A (20.9%), B (22.5%), C (25.9%) and D (30.6%) (Table 1). Total 2266 (A and B grade) oocytes were matured in maturation medium on granulosa cell monolayer for 27 h. Capacitated spermatozoa (2×10^6 spermatozoa/ml) and matured oocytes (2266) were co-incubated for 18 h. In the present study 494 embryos were produced (Table 1).

Synchronization of estrus in 14 recipient goats as carried out with two i.m. injections 10 days apart of $PGF2_{\alpha}$ (Lutalyse® 15 mg/goat). Estrus was observed 48 h after last injection. *In vitro* fertilized goat embryos (total 80) were transferred to 14 recipient goats. In each recipient 2 to 5

Table 3. Primer sequence, chromosomal location, PCR conditions and polymorphic status of the analyzed microsatellite markers

S. No	Microsatellite loci	Primer sequence	Chromosomal location	PCR condition	Polymorphic status
1	OarAE 129	AATCCAGTGTGTGAAAGACTAATCCAG GTAGATCAAGATATAGAATATTTTTCAACACC	5	Touch down	p
2	BM1314	TTCCTCCTCTTCTCTCCAAAC ATCTCAAACGCCAGTGTGG	22	,,	P
3	BM6506	GCACGTGGTAAAGAGATGGC AGCAACTTGAGCATGGCAC	1	,,	p
4	BM6526	CATGCCAAACAATATCCAGC TGAAGGTAGAGAGCAAGCAGC	26	,,	p
5	OarCP34	GCTGAACAATGTGATATGTTCAGG GGGACAATACTGTCTTAGATGCTGC	3	,,	p
6	OarCP38	CAACTTTGGTGCATATTCAAGGTTGC GCAGTCGCAGCAGGCTGAAGAGG	10	,,	p
7	ILSTS08	GAATCATGGATTTTCTGGGG TAGCAGTGAGTGAGGTTGGC	14	,,	-
8	ILSTS029	TGTTTTGATGGAACACAGCC TGGATTTAGACCAGGGTTGG	3	,,	-
9	ILSTS034	AAGGGTCTAAGTCCACTGGC GACCTGGTTTAGCAGAGAGC	5	,,	-
10	ILSTS044	AGTCACCCAAAAGTAACTGG ACATGTTGTATTCCAAGTGC	Ann	,,	p
11	ILSTS049	CAATTTTCTTGTCTCTCCCC GCTGAATCTTGTCAAACAGG	11	,,	p
12	ILSTS059	GCTGAACAATGTGATATGTTCAGG GGGACAATACTGTCTTAGATGCTGC	13	,,	p
13	OarFCB304	CCCTAGGAGCTTTCAATAAAGAATCGG CGCTGCTGTCAACTGGGTCAGGG	Ann	,,	-
14	OarJMP8	CGGGATGATCTTCTGTCCAAATATGC CATTTGCTTTGGCTTCAGAACCAGAG	6	,,	-
15	OarJMP29	GTATACACGTGGACACCGCTTTGTAC GAAGTGGCAAGATTCAGAGGGGAAG	24	,,	-

p: Microsatellite loci revealing more than three alleles. -: Microsatellite loci showing less than three alleles.
Ann: Anonymous

Table 4. Microsatellite markers, allelic size-range; number of heterozygotes and homozygotes; heterozygosity observed and expected; PIC; and exclusion probability values at each locus

S. No	Microsatellite loci	Size range (bp)	Hets	Homs	k	H (O)	H (E)	PIC	Excl (1)	Excl (2)	Null Freq.
1	OarAE 129	140-156	4	5	4	0.444	0.340	0.392	0.069	0.194	+0.000
2	OarCP34	112-126	4	5	7	0.444	0.824	0.747	0.401	0.580	+0.000
3	OarCP38	150-166	5	4	6	0.556	0.810	0.730	0.373	0.552	+0.000
4	BM6526	170-188	2	7	4	0.222	0.595	0.512	0.168	0.323	+0.000
5	BM1314	136-164	7	2	6	1.000	0.857	0.766	0.420	0.598	+0.000
6	BM6506	150-158	4	5	4	0.571	0.648	0.553	0.196	0.360	+0.000
7	ILSTS044	150-156	6	3	4	0.667	0.647	0.558	0.200	0.362	+0.000
8	ILSTS049	160-174	4	3	6	0.571	0.813	0.719	0.362	0.540	+0.000
9	ILSTS059	152-56	4	3	4	0.571	0.648	0.553	0.196	0.360	+0.000
	Mean		4.44	4.11	5	0.561	0.693	0.609	0.944	0.995	

K = Number of alleles per locus. N = Number of individuals typed for the locus. Hets = Heterozygotes. Homs = Homozygotes. H (O) = observed heterozygocity. H (E) = expected heterozygocity. PIC = polymorphic information content. Excl (1) = Exclusionary power first parent. Excl (2) = Exclusionary power second parent. Null freq = Null allele frequency estimate.

Table 5. Evaluation of paternities of the IVF kids

Offspring ID	Prob. non-exclusion	Candidate parent ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	LOD scores	Delta scores
KI	1.92E-03	D1	9	9	3	-9.49E-01	0.00E+00
KI	1.92E-03	D2	9	9	5	########	0.00E+00
KI	1.92E-03	D3	9	9	5	########	0.00E+00
KI	1.92E-03	B1	9	9	5	5.53E-01	0.00E+00*
KI	1.92E-03	B2	9	9	5	########	0.00E+00
K2	8.62E-02	D1	9	7	3	########	0.00E+00
K2	8.62E-02	D2	9	7	3	########	0.00E+00
K2	8.62E-02	D3	9	9	1	-8.89E-02	0.00E+00
K2	8.62E-02	B1	9	7	4	########	0.00E+00
K2	8.62E-02	B2	9	7	1	6.77E-01	5.88E-01*
K3	1.72E-01	D1	9	8	2	########	0.00E+00
K3	1.72E-01	D2	9	8	0	-2.74E-00	2.74E+00
K3	1.72E-01	D3	9	8	2	########	0.00E+00
K3	1.72E-01	B1	9	8	2	########	0.00E+00
K3	1.72E-01	B2	9	8	3	########	0.00E+00
K4	1.18E-01	D1	9	6	2	########	0.00E+00
K4	1.18E-01	D2	9	6	4	########	0.00E+00
K4	1.18E-01	D3	9	7	1	-1.07E-00	1.07E+00
K4	1.18E-01	B1	9	6	1	8.23E-01	0.00E+00*
K4	1.18E-01	B2	9	6	2	########	0.00E+00

O-CP loci compared = Number of loci compared between offspring and candidate parent. Mismatches = Number of alleles. which did not match between offspring and candidate parent.

embryos were transferred to uterotubal junction through laparoscopy techniques. Four kids were born (29%) (Figures 1 and 2) after completion of pregnancy in 3 goats out of 14 recipient goats. One recipient delivered twin (one male and one female) kids whereas other two recipients delivered single kid each (Table 2). One single male kid expired in severe cold weather due to pneumonia after 36 h of delivery (Figure 1).

For parentage evaluation of IVK kids. a total of 15 microsatellite markers were evaluated in the present study. Out of these. nine markers were found to be polymorphic with more than three alleles (Table 3). The remaining six loci with less than three alleles were excluded from the final analysis. Several genetic parameters estimated to determine the suitability of the microsatellite markers employed in the present investigation for parentage analysis is shown in Table 4. A total of 45 distinct alleles ranging from 4.00 (BM1314. BM6506. BM6526. ILSTS029. ILSTS034. ILSTS044. ILSTS049. ILSTS059. ILSTS08. OarFCB304 OarJMP8. OarCP34. OarCP38. OarAE129. OarJMP29 to 7.00 (Oar CP34) were detected across the 9 selected loci with a mean of 5 alleles. The allele size range observed in the studied individuals was in agreement with other studies where the similar markers were used (Saitbekova et al.. 2001: Sodhi et al.. 2003, Pandey et al., 2006). The allele size is important as in order to qualify a set of parents. an offspring must possess the same allele size as the parents. Observed and expected heterozygosity ranged from 0.222

(BM6526) to 1.000 (BM1314), and from 0.340 (OarAE129) to 0.857 (BM1314) with an average of 0.561 and 0.693 respectively. Using an iterative algorithm based on the difference between observed and expected frequencies of homozygote estimates, we estimated the frequency of null allele segregating at each locus. The estimated frequencies were close to zero for all the loci indicating absence of a null allele. It is important to note that loci with high null allele frequencies (0.05 or more) should be excluded from parentage analysis (Marshall et al., 1998). The observations reflected the suitability of markers included in the present study to evaluate the parentage of goat IVF offspring.

The genotypic profile of offspring and candidate parents were compared using the CERVUS 2.1 window programme for parentage evaluation. With the observed genotypes, a likelihood ratio for each candidate parent was calculated and compared for LOD score values (Table 5). The negative values of the LOD score implied that the each of the candidate parent (surrogate mother) is less likely to be the true parent than an arbitrarily randomly chosen individual and hence the probability of the investigated kids belonging to the surrogate mother is excluded. Buck 1 (B1) showed most positive LOD score value with kid 4 (K4) and kid (K1) genotypes whereas buck 2 (B2) had the positive LOD score for kid 2 (K2). This analysis revealed buck 1 as most likely parent for kids 4 and 1 whereas buck 2 was the most probable parent for kid 2.

To evaluate the significance of assigned paternities the data was simulated 10 thousand times. From the distribution of the log likelihood statistic delta values it was observed that the buck 1 had a delta score exceeding the critical delta score for 95% confidence for two kids and hence is true parent for them. Buck 2 had a delta score exceeding the critical delta score for 95% confidence only for one kid.

DISCUSSION

Goat embryos were produced through *in vitro* maturation, fertilization and culture with the oviductal cells. In the present study, 5,212 oocytes were collected from 1,977 ovaries and 2.63 oocytes per ovary were obtained (Table 1) which was lower than 6.04 (Keskintepe et al., 1994) and 3.41 (Malakar and Majumdar, 2005) and more than 2.28 (Pawshe et al., 1994) as collected from follicles 2-8 mm. Though direct maturation was not studied in the present work but cumulus expansion was taken as the indication of the maturation of oocytes. Healthy, culturable with cumulus cell complexes A (20.9%) and B (22.5%) grade oocytes were matured on granulosa cell monolayer that showed good cumulus expansion (Teotia et al., 2001). The cumulus cells secret a meiosis inducing substance that increases the maturation rate of oocytes (Xia et al., 1994; Karina and Charlotte, 2004). Concentration of processed spermatozoa helped in controlling polyspermy (De Smedt et al., 1992) during sperm-egg interaction. Younis et al. (1991) reported 68% fertilization using 1×10^6 sperm/ml with 5 matured oocytes in 100 µl fertilization TALP medium. Pawshe et al. (1995) also used 2×10^6 sperm/ml of medium for 10 oocytes. In present study, sperm concentration was 2×10^6 sperm/ml and 10 to15 oocytes were added per drop. The duration of sperm-egg incubation has reported many workers as 17 h (Martino et al., 1995), 16 to 18 h (Brackett et al., 1989), 20 to 22 h (Herrick et al., 2004) and 24 h (Keskintepe et al., 1994) however, in the present study, sperm-egg incubation period was 12 h.

Wide variation of *in vitro* cleavage rates has been reported to be 23.1% (Malakar and Majumdar, 2002), 33.3% (Younis et al., 1991), 42.8% (Izquierdo et al., 2002), 26% (Crozet et al., 1993), 40% (Pawshe et al., 1995), 42% (Han et al., 2001) and 79.4% (Kastka et al., 2004) respectively by various workers. In the present study embryos cultured in medium supplemented with L-glutamine alone with TCM-199 on goat oviductal cells yielded 21.8% cleavage (Table 1). The cleavage rate was less in comparison to other studies due to the fact that all A and B grade oocytes were used in IVF without any selection.

Ruth-Macedo et al. (1999) reported that synchronization of estrus with $PGF2_\alpha$ (16 mg/goat, 2 injections 10 days apart), gonadotropin releasing hormone (0.0126 mg) and an ear implant of progestogen resulted in observed oestrus in 100, 67 and 50% respectively in Criollo goat. Tamboura et al. (1997) have reported 2 i.m. injections of $PGF2_\alpha$ (Estrumate) 12 days apart and oestrus occurred 67±5 h after treatment. In the present study synchronization of estrus was carried out with $PGF2_\alpha$ (15 mg/goat, 2 i.m. injections 10 days apart) and oestrus was observed in all goats after 48 h of last injection and results were comparable to those obtained by Ruth-Macedo et al. (1999).

Hanada (1985) produced the first goat kids using the IVF procedure on ovulated oocytes in the goat. Pregnancy was initiated in goats by embryo transfer after *in vitro* maturation and fertilization of goat oocytes (Younis et al., 1991). The first birth of a kid from an oocyte matured and fertilized *in vitro* was performed Crozet et al. (1993). Surgically transferred of 12 IVF embryos at 6 to 8 cell stage to synchronized 2 recipients and both of the recipients became pregnant. One recipient miscarried while the other gave birth to female normal twins (50%) (Katska et al., 2002). Forty 2 to 4 cell IVF goat embryos were transferred to 20 recipients does after 48 h of incubation and 4 single kids were born (20%) (Bou-ShorGan, and Bou-SG, 1998). Han et al. (2001) reported that 6 viable kids were born (31%) from 30 good quality embryos transferred to 19 recipient goats. Thirty-seven IVF transgenic embryos were transferred to 32 recipient goats and four live kids were produced (Huang et al., 2001). Baldassarre et al. (2004)

used laparoscopic ovum pick up technique followed by *in vitro* embryo production in late prepubertal and younger age resulting in 27 and 15 kids respectively. In the present study, 80 IVF goat embryos were transferred to 14 recipient goats and 4 kids were born (29%) in 3 recipient goats (Table 2). The results are similar to those obtained by Bou-ShorGam and Bou-SG (1998) and Han et al. (2001).

In the present investigation, panel of polymorphic microsatellite markers recommended by FAO for biodiversity analysis were evaluated for assigning parentage. The high exclusionary power of such a battery of genetic markers shows the utility of such markers for evaluating the parentage (Isberg et al., 2004, Kong et al., 2006; Osman et al., 2006). The parentage assignment at a known level of statistical confidence (95%) clearly showed that the kids borne are the IVF kids. The use of these codominant microsatellite markers will broaden the scope of *in vitro* fertilization program, allowing progeny to be tested from adults maintained in herd.

In conclusion, *in vitro* maturation and fertilization of goat oocytes was carried out from slaughterhouse ovaries. Cleaved embryos were co-cultured with goat oviductal epithelial cells. Total 80 embryos were transferred using simple technique of laparoscopy to 14 recipient goats and four live kids were born (29%) in 3 recipients (Table 2). To best of our knowledge, this is the first report in India about producing live normal offspring in Indian breeds of goats using IVF procedures followed by the transfer of embryos using very simple laparoscopy technique and their parentage was confirmed using microsatellite markers.

REFERENCES

Baldassarre, H., B. Wang, N. Kafidi, C. L. Keefer, A. Lazaris and C. N. Karatzas. 2002. Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. Theriogenol. 57:275-284.

Baldassarre, H., B. Wang, J. Pierson, N. Neveu, L. Sneek, J. Lapointe, F. Cote, N. Kafidi, C. L. Keefer, A. Lazaris and C. N. Karatzas. 2004. Prepubertal Propagation of Transgenic Cloned Goats by Laparoscopic Ovum Pick-Up and *In vitro* Embryo Production. Cloning and stem cell 6:25-29.

Bassam, B. J., G. Caetano-Anolles and M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196:80-83.

Bou-ShorGan and S. G. Bou. 1998. Application of *in vitro* fertilization and embryo transfer technology in farm animals in Inner Mongolia, China. J. Reprod. Develop. 44:j61-j69.

Brackett, B. G., A. I. Younis and R. A. F. Hosten. 1989. Enhanced viability after IVF of bovine oocytes matured *in vitro* with high concentrations of LH. Fertil. Steril. 52:319-324.

Cognie, Y., G. Baril, N. Poulin and P. Mermillod. 2003. Current status of embryos technologies in sheep and goat. Theriogenol. 59:171-188.

Cognie, Y., N. Poulin, Y. Locatelli and P. Mermillod. 2004. State-of-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. Repro. Fertil. Dev. 16:437-445.

Cowan, C. A., J. Atienza, D. A. Melton and K. Eggan. 2005. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Sci. 309:1369-1375.

Crozet, N., V. De Smedt, M. Ahmed-Ali and C. Sevellec. 1993. Normal development following *in vitro* oocyte maturation and fertilization in the goat. Theriogenol. 39:206 (Abstr.).

De Smedt, V., N. Crozet, M. Ahmed-Ali, A. Martino and Y. Cognie. 1992. *In vitro* maturation and fertilization of goat oocytes. Theriogenol. 37:1049-1060.

Fan, B., Y. Z. Chen, C. Moran, S. H. Zhao, B. Liu, M. Yu, M. J. Zhu, T. A. Xiong and K. Li. 2005. Individual-breed Assignment Analysis in Swine Populations by Using Microsatellite Markers. Asian-Aust. J. Anim. Sci. 18(11): 1529-1534.

Food and Agricultural Organization of the United Nations (FAO). Global projects for the maintenance of Domestic Animal Genetic Diversity (MoDAD).1996. http://www.fao.org/dad-is/, FAO, Rome.

Han, Y., M. Meintjes, K. Graff, R. Denniston, L. Zhang, C. Ziomek and R. Godke. 2001. Caprine offspring born from fresh and frozen-thawed *in vitro*-produced embryos. Vet. Rec. 149:714-716

Hanada, A. 1985. *In vitro* fertilization in goat. Jpn. J. Anim. Reprod. 31:21-26.

Herrick, J. R., E. Behboodi, E. Memili, S. Blash, Y. Echelard and R. L. Krisher. 2004. Effect of macromolecule supplementation during *in vitro* maturation of goat oocytes on developmental potential. Mol. Reprod. Dev. 69:338-346.

Huang, S. Z., Y. Huang, M. J. Chen, F. Y. Zeng, Z. R. Ren and Y. T. Zeng. 2001. Selection of *in vitro* produced, transgenic embryos by nested PCR for efficient production of transgenic goats. Theriogenol. 56:545-556.

Hunter, G. L., C. E. Adam and L. E. Rowse. 1995. Inter-breed ovum transfer in sheep. J. Agril. Sci. 46:143-149.

Isberg, S. R., Y. Chen, S. G. Barker and C. Moran. 2004. Analysis of Microsatellites and Parentage Testing in Saltwater Crocodiles. J. Hered. 95:445-449.

Izquierdo, D., P. Villamediana, M. Lopez-Bejar and M. T. Paramio. 2002. Effect of *In vitro* and *in vivo* culture on embryo development from prepubertal goat IVM-IVF oocytes. Theriogenol. 57:1431-1441.

Karina, F. R. and E. F. Charlotte. 2004. Gene transcription and regulation of oocyte maturation Reprod. Fertil. Dev. 16(2): 55-67.

Katska, L., B. Rynska, B. Gajdaand and Z. Smorag. 2004. Effect of donor stimulation, frozen semen and heparin treatment on the efficiency of *in vitro* embryo production of goats. Theriogenol. 62:576-586.

Katska, L., G. Kania, B. Rynska, B. Gajda and Z. Smorag. 2002. Offspring after transferring goat embryos originating from *in vitro* matured and fertilized oocytes. Medycyna-Weterynaryjna. 58:462-463.

Keskintepe, L., G. M. Darwish, A. T. Kenimer and B. G. Brakett. 1994. Term development of caprine embryos derived from immature oocytes *in vitro*. Theriogenol. 42:527-535.

Kong, J. H., S. D. Oh, J. H. Lee, K. J. Jo, B. D. Sang, C. H. Choi, S. D. Kim, S. J. Lee, S. H. Yeon, G. J. Jeon and H. K. Lee. 2006. Genetic Variation and Relationships of Korean Native Chickens and Foreign Breeds Using 15 Microsatellite Markers. Asian-Aust. J. Anim. Sci. 19(11):1546-1550.

Litt, M. and J. A. Luty. 1989. A hypervarible microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am. J. Hum. Genet. 44:397-401.

Malakar, D. and A. C. Majumdar. 2002. Secretory proteins from goat oocytes matured in culture. Asian-Aust. J. Anim. Sci. 15:340-345.

Malakar, D. and A. C. Majumdar. 2005. Isolation, identification and characterization of secretory proteins of IVMFC embryos and blood circulation of estrus and early pregnant goat. Indian J. Expt. Biol. 43:693-701.

Marshall, T. C., J. Slate, L. Kruuk and J. M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7(5):639-655.

Martino, A., T. Mogas, M. J. Palomo and M. T. Paramio. 1995. *In vitro* maturation and fertilization of prepubertal goat oocytes. Theriogenol. 43:473-485.

O'Brien, J. K., F. K. Hollinshead and W. M. C. Maxwell. 2004. *In vitro* development capacity of *in vitro* produced embryos derived from sex-sorted and re-cryopreserved frogen-thawed ram sperm. Repro. Fertil. Dev. 16:286-290.

Oppenheim, S. M., A. L. Moyer, R. H. BonDurant, J. D. Rowe and G. B. Anderson. 2000. Successful pregnancy in goats carrying their genetically identical conceptus. Theriogenol. 54:629-639.

Osman, S. A. M., M. Sekino, A. Nishihata, Y. Kobayashi, W. Takenaka, K. Kinoshita, T. Kuwayama, M. Nishibori, Y. Yamamoto and M. Tsudzuki. 2006. The Genetic Variability and Relationships of Japanese and Foreign Chickens Assessed by Microsatellite DNA Profiling. Asian-Aust. J. Anim. Sci. 19 (10):1369-1378.

Pawshe, C. H., S. M. Totey and S. K. Jain. 1994. A comparison of the three methods: of recovery of goat oocytes for *in vitro* maturation and fertilization. Theriogenol. 42:117-125.

Pawshe, C. H., M. Taneja, S. K. Jain and S. M. Totey. 1995. *In vitro* maturation, fertilization and development of goat follicular oocytes. In: proceeding of the 1995 Xth Annu. Conf. SOC. Anim. Physiol. Ind. (SAPI). 23-25 Feb., CARI, Izatnagar p. 86 (Abstr.).

Reed, W. A., T. K. Suh, T. D. Bunch and K. L. White. 1996. Culture of *in vitro* fertilized bovine embryos with bovine oviductal epithelial cells. Buffalo, rat liver cells (BRL) or BRL-cell conditioned medium, Theriogenol. 45:439-445.

Ruth-Macedo, M., A. C. Vega, O. R. Wilde and A. C. Dela-Vega. 1999. Evaluation of hormonal treatments in Crillo goats during the spring season. Vet-Argentina 156:438-445.

Pandey A. K., Rekha Sharma, Yatender Singh, B. Prakash and S. P. S. Ahlawat. 2006. Evaluation of Genetic Variability in Kenkatha Cattle by Microsatellite Markers. Asian-Aust. J. Anim. Sci. 19(12):1685-1690.

Saitbekova, N., C. Gaillord, G. Obexer–Ruff and G. Dolf. 1999. Genetic diversity in Swiss goat breeds based on microsatellite analysis. Anim. Genet. 30:36-41.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular Cloning:In: A laboratory manual. Cold Spring Harbour Lab. Press. Cold Spring Harbour. NY.

Shin, S. T., S. K. Jang, H. S. Yang, O. K. Lee, Y. H. Shim, W. I. Choi, D. S. Lee, J. K. Cho and Y. W. Lee. 2004. 182 laparoscopic embryo transfer in Korean black goats. Reprod. Fertil. Dev. 16(2):213-213.

Sodhi, M., M. Mukesh, A. Anand, S. Bhatia and B. P. Mishra. 2006. Assessment of Genetic Variability in Two North Indian Buffalo Breeds Using Random Amplified Polymorphic DNA (RAPD) Markers. Asian-Aust. J. Anim. Sci. 19(9):1234-1239.

Sodhi, M., M. Mukesh, R. Arora, M. S. Tantia and S. Bhatia. 2003. Genetic structure of Garole- a unique Indian microsheep assessed using microsatellite markers. Indian J. Dairy Sci. 56(3):167-173.

Sukla, S., B. R. Yadav and T. K. Bhattacharya. 2006. Characterization of Indian Riverine Buffaloes by Microsatellite Markers. Asian-Aust. J. Anim. Sci. 19(11): 1556-1560.

Tamboura, H. H., L. L. Sawadogo, C. Tahiri, D. Aidara and A. D. E. Bogore. 1997. Estrus synchronization and post-partum management of "Mossi" local breed of goat in Burkina Faso. Tropicultura 15:190-194.

Tecirlioglu, R. T., A. J. French, I. M. Lewis, G. Vajta, M. A. Korfiatis, V. J. Hall, T. N. Ruddock, M. A. Cooney and A. O. Trounson. 2003. Birth of cloned calf derived from a vitrified hand-made cloned embryo. Reprod. Fertil. Dev. 15:361-366.

Teotia, A., G. T. Sharma and A. C. Majumdar. 2001. Fertilization and development of caprine oocytes matured over granulosa cell monolayers. Small Rum. Res. 40:165-177.

Xia, G. A., G. Byskov and C. Y. Andersen. 1994. Cumulus cells secrete a meiosis-inducing substance by stimulation with forskolin and dibutyric cyclic adenosine monophosphate. Mol. Reprod. Dev. 39:17-24.

Younis, A. I., K. A. Zuelke, K. M. Harper, M. A. L. Oliveira and B. G. Brackett. 1991. *In vitro* fertilization of goat oocytes. Biol. Reprod. 44:1177-118.