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Birth of twin kids following transfer of *in-vitro* produced goat embryos

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Post-implanted growth and development of embryo is deficient in many aspects as evidenced by the great difficulty in growing embryos, while maintaining viability as shown by development of early embryos *in-vitro* generally delayed or completely blocked at 8–16 cell stage embryo, and hardly small percentage of the *in-vitro* fertilized embryo develop to morula and blastocyst (Kharche *et al.* 2008b). Investigations on *in-vitro* embryo production and to assess the post transfer survival of *in-vitro* produced embryos in goats have their usefulness for both basic research and commercial application. Therefore, the present study was aimed to evaluate *in-vitro* embryo development potential and to asses post transfer survival rate of *in-vitro* produced embryos.

Collection of ovaries: Ovaries of goats were obtained from slaughterhouse located at Agra. Ovarian tissues were transported to the laboratory in a thermos flask containing sterile and warm physiological normal saline solution (NSS) supplemented with antibiotics (100 µg/ml penicillin-G and 100 µg/ml streptomycin sulphate). Prior to oocytes retrieval, surplus tissues on the ovaries were removed and ovaries were washed several times in normal saline containing antibiotics to avoid any form of microbial contamination.

Recovery of oocytes and in-vitro *maturation (IVM):* The oocytes were collected from each ovary in oocyte collection media (OCM: Dulbecco's phosphate-buffered saline with 3 mg/ml BSA, 50µg/ml streptomycin and 60µg/ml penicillin) by follicle puncture method using 18-G needle. Isolated oocytes were washed 4-to-5 times in oocyte holding medium (OHM) containing (TCM-199 with Hepes modification, EGS 10%, sodium pyruvate 0.25 mM, gentamycin 50 µg/ml, glutamine 100 µg/ml, BSA 3 mg/ml) and graded. The oocytes were then washed 3-times with oocyte maturation medium (OMM) containing (TCM-199 supplemented with 10% FBS, sodium pyruvate 0.25 mM, glutamine 100 µg/ml, LH 10 µg/ml, FSH 5µg/ml, BSA 3 mg/ml and gentamycin 50µg/ml) and were allowed to mature in 50µl droplets of IVM medium for 24 –27 h in a CO_2 incubator maintained at 38.5°C, 5% CO_2 and high humidity.

In-vitro *fertilization:* After 27 h of *in-vitro* maturation oocytes were denuded by hyaluronidase enzyme and by passing repeatedly through fine pipette. The oocytes were washed 8–10 times in Fert TALP (TALP solution containing 10% oestrous goat serum, 8 mg/ml fatty acid free BSA and 100 µg / ml heparin) and put in 50 µl drop in culture petri-dish. Fertilization drops containing oocytes were inseminated with 25 to 50 µl of finally diluted semen so as to obtain a sperm concentration of 2–4 millions sperm /ml. The sperm-oocytes were co-incubated for 18 hr in a CO_2 incubator maintained at 38.5° C, 5% CO_2 and high humidity.

In-vitro *embryo development:* Following 18 hr of sperm oocytes co-incubation, the oocytes were washed in KSOM medium containing 10% FBS and 3 mg/ml BSA and then cultured in 50 µl drop of embryo development medium. The zygotes were cultured under mineral oil in 5% CO_2 in air with high humidity at 38.5°C. After 48h of post insemination, oocytes were observed for cleavage and half of the culture medium was replenished with fresh medium. Embryos were morphologically evaluated under inverted phase contrast microscope to separate retarded embryos from normally developing embryos.

Embryo transfer and pregnancy diagnosis: In-vitro produced embryos were transferred in Sirohi goats at natural oestrus. Recipient was deprived of feed and water for 24 h and put under general anesthesia using xylazine (0.2 mg/kg body weight) and ketamine (4.4 - 6.6 mg/kg body weight). The reproductive tract was exteriorized through a mid-ventral incision to allow visual confirmation of a corpus luteum/ lutea on ovaries. Morphological embryos were grouped under 2 category i.e. early stage embryos and late stage embryos. Early stage embryos (2 to 4 cell) were transferred surgically into the fallopian tube ipsilateral to the ovary containing corpus luteum of 2 naturally synchronized surrogate doe (8 embryos each). Similarly, 8–16 cell and morula stage embryos were transferred surgically at the tip of uterine horn ipsilateral to the ovary containing corpus luteum of 6 naturally synchronized surrogate doe (8 embryos each).

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Fig. 1. Twin IVF kids.

Following transfer, 2 recipients were initially found pregnant at day 35th post transfer by ultrasonography. Subsequently on re-examination at day 60, one recipient was confirmed pregnant and delivered twin offspring of male and female sex after 142 days of gestation.

Morphology of the cytoplasm and cumulus cell investment surrounding oocytes is the primary criteria for the grading and selection of oocytes for IVM. Normal oocytes should have cumulus cell investment surrounding the zona pellucida (ZP), absence of cracked zona pellucida and absence of vesicle in ooplasm. The recovery of good quality oocytes was 2.12/ovary. The results showed that immature oocytes collected from ovaries of slaughtered goats can be fertilized and cultured up to the blastocyst stage. The average percentage of immature oocytes reaching the cleavage, 2 cell, 4 cell, 8–16 cell, morulae and blastocysts were 44%, 17%, 25%, 33.11%, 16% and 2%, respectively. Further, the method to evaluate post - transfer survival rate was documented by successful birth of live offspring following transfer of early stage (2–4 cell stage embryos) and late stage embryos (8–16 cell and morulae) into the fallopian tube and uterine horn of naturally synchronized recipients, respectively. Keskintepe *et al.* (1996) also reported the birth of 2 normal female kids after transfer of morule into the uterine horn ipsilateral to the corpus luteum of a recipient and established feasibility for direct uterine transfer of caprine embryos produced by IVMFC.

Recent advances in the field of biotechnology and allied sciences for the production of goat embryos has led to development of several media, additives and culture conditions to optimize *in-vitro* embryo development (Kharche *et al.* 2008b). Major developmentally important events take place during development of embryos from post fertilization to the blastocyst stage. The blastocyst stage of embryos show more chances of survival and are able to produce live offspring with higher success of birth rate. Defined culture media have been designed to more exactly fit embryo requirements without the need for co-culture as it negates the possible effects of unknown components, minimizes risk of contamination with pathogens, reduces variations among different lots of media and prevents variation among different cultures batch by the use of biological fluids and co-culture system. Good development of embryos can be obtained in a completely defined medium as compared with complex culture medium (Kharche *et al*. 2010). Potassium simplex optimized medium (KSOM) were found capable of supporting the development of cattle zygotes to the blastocyst stage even in the absence of co-culture with somatic cells (Westberg *et al.* 2002). In our previous study, we also observed higher (31.4%) cleavage rate in KSOM medium as compared to SOF and TCM medium. Papadopoulos *et al.* (2002) and Malakar *et al*. (2007) reported a conception rate of 54.3% and 21.4% following uterine transfer of *in-vitro* produced goat blastocyst and morula, respectively. In our earlier study a conception rate of 20.00% was achieved following transfer of 8 to 16-cell stage *in-vitro* produced embryos at the tip of the uterine horn (Kharche *et al*. 2008a). Katska-Ksiazkiewicz *et al.* (2004) studied post transfer survival of *in-vitro* produced caprine embryos and reported more than 60% of the recipients became pregnant and 20% of all transferred embryos survived delivering 13 healthy kids. In the present study we observed the post transfer survivability of *in-vitro* produced embryos cultured in defined media. We transferred early stage embryos (2–4 cell stage) into the oviduct of naturally synchronized recipients and late stage embryos (8–16 cell stage to morula stage) into the uterus of naturally synchronized recipient. Present work represents the successful birth of twin IVF kid with conception rate of 12.5%. Post-implanted growth and development of embryo is deficient in many aspects as is evidenced by the great difficulty in growing embryos while maintaining viability as shown by development of early embryos *in-vitro* generally delayed or completely blocked at 8–16 cell stage embryo and hardly small percentage of the *in-vitro* fertilized embryo develop to morula and blastocyst, which is probably due to minor physio- chemical variation and lack of certain factors in the culture system. Improvements in IVMFC procedure enabled consistent IVP of goat uterine stage embryos.

SUMMARY

Oocytes (1127) were collected from 531 goat ovaries of abattoir origin. Collected oocytes were matured in TCM-199 medium containing 5µg/ml FSH, 10µg/ml LH, 10% FBS and 3 mg/ml BSA at 38.5°C in CO_2 incubator under humidified air. After 27 h of *in-vitro* maturation oocytes were denuded by hyaluronidase enzyme and by passing repeatedly through fine pipette. After 48h post insemination, oocytes were observed for cleavage. Cleavage rate, 2 cell, 4 cell, 8–16 cell, morulae and blastocysts were 44%, 17%, 25%, 33.11%, 16% and 2%, respectively. *In-vitro* produced

embryos of 2 - 4 cell stage were surgically transferred into the fallopian tube ipsilateral to the ovary containing corpus luteum of two naturally synchronized surrogate does (8 embryos each). Similarly, 8–16 cell and morula stage embryos were transferred surgically at the tip of uterine horn ipsilateral to the ovary containing corpus luteum of 6 naturally synchronized surrogate does (8 embryos each). Following transfer, 2 recipients were initially found pregnant at day 35th post transfer by ultrasonography. Subsequently on re-examination at day 60, only 1 recipient was confirmed pregnant and delivered twin offspring of male and female sex after 142 days of gestation.

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