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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(3): 11-14
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www.thepharmajournal.com
Received: 10-01-2021
Accepted: 13-02-2021

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In-vitro embryo production in caprine using slaughterhouse ovaries

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DOI: https://doi.org/10.22271/tpi.2021.v10.i4Sa.5950

Abstract
The slaughterhouse Caprine ovaries were the cheap source of Caprine germplasm, and it was collected in a hygienic method within a short period. The slicing was the method of oocyte isolation followed in this study. The grade A and B quality oocytes were isolated, washed, and matured in a prepared media. The does in standing heat was identified and fresh buck semen was collected from trained buck available in the animal shed. The collected semen was evaluated for physical attributes, mass motility, live percentage of spermatozoa, individual motility, acrosomal integrity, and the Hypoosmotic swelling test (HOST). The semen from proven buck was used for the successful in-vitro fertilization (IVF) of oocyte and production of Caprine embryos. Various factors were affected the successful production of cleaved embryos in Caprine and were well explained by various authors. The stepwise precautions needed to be taken to successful *in vitro* embryo production were the main objective of this study.

Keywords: IVEP, caprine, IVF, semen, ovaries

Introduction
The *in vitro* embryo production (IVEP) and embryo transfer in small ruminants have the potential to produce large number offspring, and capable to bridge demand-supply gap in the meat industry in developing countries. The protein requirement of Indian population can be meet through chevon and goat milk production taking Compound Annual Growth Rate (CAGR) by 5 and 7% respectively by 2022-23 (DAHD, 2017, NAP on goat 2022) [4]. The *in vitro* produced embryos are cheap source of embryo for fundamental and applicable level research as well as conservation endangered germplasm can be attained through IVEP (Baldasree *et al.*, 2002) [1] (Ptak *et al.*, 2002) [2].

The successive *in vitro* embryo production in caprine species depends on various factors in each stage of embryo production. The oocyte recovery or ovum pick up, *in vitro* maturation of oocytes, semen collection and preparation, *in vitro* fertilization and *in vitro* culture of embryos are the main stages of IVEP. The factors like composition of collection, maturation, fertilization and culture media have significant impact on the cleavage rate of embryos (Cognie *et al.*, 2003) [3]. Other than this incubation conditions like temperature, humidity and percentage of CO_2 in each step of embryo production have intricate influence on IVEP.

The time between doe slaughter and isolation of oocytes from slaughtered doe was minimal, and the transport of ovary from slaughter house to lab was in sterile conditions in prewarmed transport media. The method of oocyte isolation was slicing. A large number of oocytes were retrieved and handling of small sized Caprine ovaries were made slicing superior than other oocyte isolation method. The components of IVM media, and supplementation of additives like cysteamine, melatonin, estrus goat serum, new calf serum, and Caprine follicular fluid have impact on cleavage rate of goat embryos. The quality and quantity of semen plays a major role in the success of IVF with the quality of oocytes. The effect of granulosa cells and oviductal epithelial cells on embryo development were studied by various researchers.

While, researchers have been stressed the importance of quality oocytes and media compositions for successful Caprine embryo development, the Caprine semen attributes have been given less importance in many studies. In this aforementioned situation, this study given equal importance to male and female counterparts for successful Caprine embryo production.

Material and Methods

Oocyte isolation

The chemicals used in this study were procured from Sigma-Aldrich unless separately mentioned. Caprine ovaries were transported from an abattoir to the laboratory in normal saline (NS) containing antibiotics pencillin (60mg/L) and streptomycin (50mg/L). The ovaries were washed twice using NS in lab. The ovaries were sliced using BP blade in a petri dish (90mm) containing Oocyte isolation media (DPBS-1pouch/L, Dextrose- 1g, Sodium Pyruvate-36mg, Calcium chloride- 100mg, Streptomycin-50 mg, Pencillin- 60mg, Bovine Serum Albumin (BSA)- 1mg/mL). The grade A & B quality Cumulus oocytes complex (COCs) were picked and kept for *in vitro* maturation (IVM) at 38.5 °C and 5% CO_2.

In-vitro maturation (IVM) of oocytes

The isolated COCs were kept for IVM in media (TCM199, 10% FBS, Follicle Stimulating Hormone (FSH)- 5µg/mL, Luteinizing Hormone (LH)- 10 µg/mL, Estradiol- 1 µg/mL, Glutamine-0.1mg/mL, Sodium pyruvate- .25mM and BSA-3mg/mL) for 27 hrs at 38.5 °C and 5% CO_2.

Semen Preparation and evaluation of semen attributes

The semen collected from a healthy Sirohi buck using artificial vagina (AV). The semen attributes were checked. The mass activity of the semen sample was determined by placing a drop of fresh semen on clean, grease free glass slide without cover slip and mounted on a stage maintained at 37 °C under low power of microscope. It was graded on the scale of 0 to +5 according to Salisbury *et al.,* 1978 [8]. The motility was recorded as percentage of progressively motile spermatozoa after the extension of a drop of fresh semen with 100 µL of Tris dilutor and observed under high power objective (400x) after putting cover slip over it. The semen sample was diluted so that approximately15 to 20 spermatozoa were visible under the visual field of microscope. The percentage of viable spermatozoa was estimated by vital staining technique using Eosin- Nigrosin stain (Swanson and Bearden, 1951) [6]. A drop of Eosin-Nigrosin stain (1:3 drops mixture) was taken on clean, grease free pre warmed glass slide to which one drop of semen was added, and mixed quickly but gently using a blunt fine glass rod. After 30 s, a thin smear was made on the same slide and examined under oil immersion objective of Bright field microscope (Motic, China). A total of about 200 spermatozoa were counted and per cent value calculated for each slide. Both fully stained and partially stained spermatozoa were considered as dead/ damaged. The acrosome integrity of spermatozoa was studied in Giemsa-stained smears according to method described by Watson (1975) [5]. The acrosomes manifesting marked swelling, knobbing, ruffling, or incomplete contour and denudation were recorded as abnormal. The HOS test was performed as per the method described by Jeyendran (1984) [7] to assess the functional integrity of plasma membrane of spermatozoa. 100µL of fresh semen washed twice with spermTALP (Table. 1 & 2) at 1200 rpm for 5 minutes. Then the sperm pellet was washed with capacitation media and 100µL of sperm pellet was pipetted into 900µL of capacitation media.

In-vitro fertilization and *In-vitro* culture

The matured oocytes were washed and transferred to a fertilization media (FerTALP, Table. 1 & 2) and capacitated spermatocytes were transferred to the same drop of fertilization media. The matured oocytes were denuded using 0.1% hyaluronidase (Sigma, H2126). The spermatocytes and oocytes were co-incubated for 18 hrs at 38.5 °C and 5% CO_2. The presumed zygotes were washed and transferred to the RVCL50 media (Cook Medical, K-RVCL-50) media for further culture at 38.5 °C and 5% CO_2 for 8-9 days.

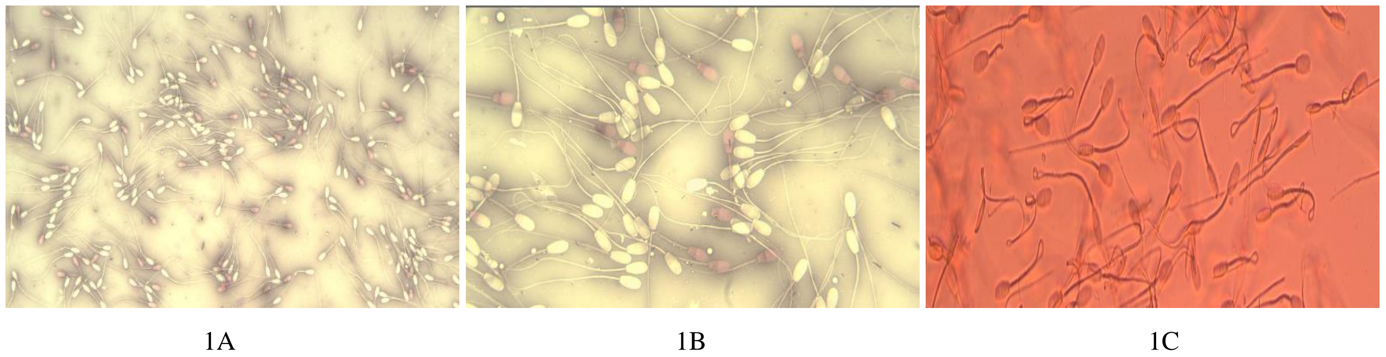
Results and Discussion

Evaluation of fresh buck semen

The Sirohi buck semen was collected using AV which covered in insulated jacket, and the graduated collection glass tube kept in 37 °C to maintain the activity of spermatozoa. The volume, colour and consistency of fresh buck semen was 1 mL, white and creamy respectively. The mass motility was ≥4+ in 0 to 5 scale, and the individual progressive motility was noted as 87%. The sperm concentration was 3550 million/mL of semen and percentage of live spermatozoa was 89. The ejaculate had ≥ 90% acrosome intact, and >70% HOST positive spermatozoa (Fig. 1A-1E). The collection of semen in prewarmed AV set was important. The preparation of AV was done in shed in a hygienic condition. The identification of animal with standing heat was critical in the successful semen collection procedure (Leboeuf *et al.,* 2000) [9]. The buck was healthy and mineral supplementation was included in the daily ration. The sexual rest of buck in breeding season is very much important for successful collection of good quality semen (Boue and Corteel, 1992) [10]. The handling of buck, doe and semen collection protocol were completed by a trained attender available in the facility.

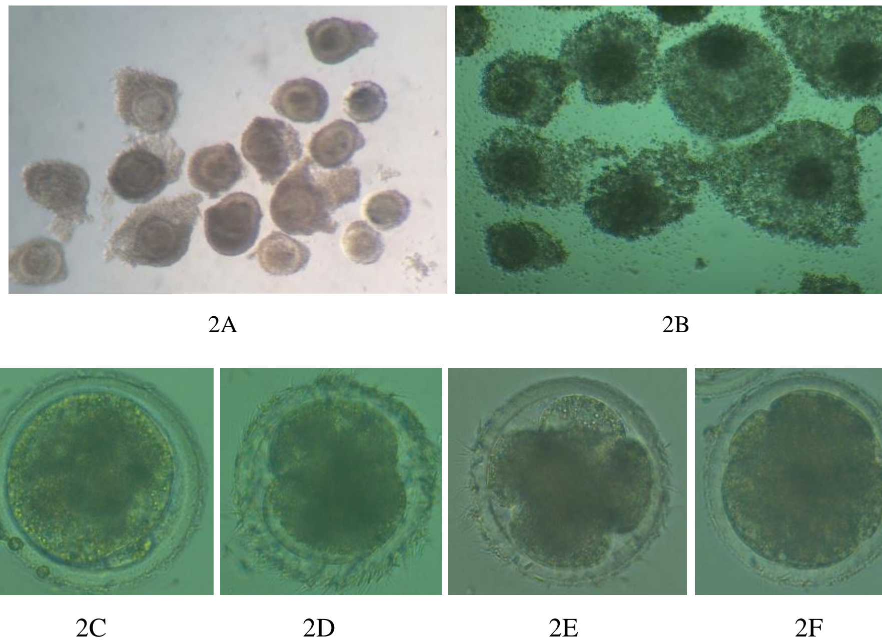
In vitro maturation and *in vitro* fertilization

The oocytes collected per ovary was 8. The ovaries with large CL were discarded. Percentage of matured oocytes per collection was 70% and percentage of cleaved embryos 55.55% (Fig. 2A-2F). The sterility of normal saline in which ovaries were transported and oocyte isolation media was maintained by the addition of antibiotics. The prewarmed saline and media used for transport of ovary and isolation of oocytes. The ovaries were trimmed to avoid unwanted tissue debris and washed twice in prewarmed saline. The very large follicles were aspirated and then the ovaries were sliced using suitable size (21) of BP blade. While slicing the ovaries, the tissue debris was minimal and the rupture of large follicle in media was completely avoided. The rupture of large follicle releases large volume of follicular fluid which contains various factors that can clot the oocyte isolation media. 50mL of oocyte isolation media was used to slice 3-4 ovaries based on the transparency of media after slicing of each ovary. Within short time, the grade A and B quality of oocytes were isolated, washed in a wash media and kept for IVM (Kharche *et al.,* 2008) [11]. The IVM media was pre-incubated at 38.5°C for 2 hours, and 10-15 oocytes were placed in a 75 µL of IVM media. The expansion of cumulus oocyte complex was taken as indication of oocyte maturation. The capacitated semen was pipetted into oocyte containing *in vitro* fertilization media drop up to circling movement of oocytes by spermatozoa. The excess concentration of spermatozoa was avoided due to uneasy recovery of oocyte from the IVF media after co-incubation. Factors influencing successful Caprine embryo production well explained by Kharche and co-workers (Kharche *et al.,* 2011) [12].



1A 1B 1C

Fig 1A: live and dead spermatozoa (400x), 1B; live and dead spermatozoa (1000x), 1C; HOST (400x), 1D; Acrosomal integrity (400x), 1E; Acrosomal integrity (1000x).



2A 2B

2C 2D 2E 2F

Table 1: Stock solution for preparation of SpermTALP and FerTALP

S. No.	Stock	Components	For 100mL
1.	Stock A	NaCl NaH_2PO_4 KCl Gentamycin	6.667g 0.062g 0.238g 50µg
2.	Stock B	$NaHCO_3$	0.84g
3.	Stock C	Sodium Pyruvate	0.036g/10mL
4.	Stock D	$CaCl_2.2H_2O$	0.252g/10mL
5.	Stock H	HEPES free acid HEPES sodium salt	.3g/10mL .35g/10mL
6.	Stock L	Sodium Lactate	.47/9.53mL of DW
7.	Stock M	$MgCl_2.6H_2O$	.1g/10mL

Table 2: Composition of SpermTALP and FerTALP (Heparin 10 µg/mL) should add to FerTALP to make capacitation media)

S. No.	Components	FerTALP (20mL)	SpermTALP (20mL)
1.	Water	14.4mL	16.3mL
2.	FBS	1	2.5
3.	Stock A	2	2.5
4.	Stock B	2	.2
5.	Stock C	.16	.2
6.	Stock D	.24	.3
7.	Stock H	-	1.5
8.	Stock L	.6	.750
9.	Stock M	.2	.250
10.	Antimycotic	10µL/mL	10µL/mL
11.	BSA	8mg/mL	6mg/mL

Author contributions

All authors contributed to the work. MS and BJ conceptualized the work. BJ, AK and MKT did the lab work and prepared manuscript. Other members helped the editing of the manuscript.

Acknowledgements

None.

Funding

Part of NASF project received by Dr. Mihir Sarkar

Conflict of interest

All authors declared that there is no conflict of interest

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