



## A comparison of *in vitro* fertilization potential of sperm obtained from refrigerated (4°C) epididymis and fresh ejaculation of buck under different embryo culture media

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### ABSTRACT

The present study assessed the fertilization potential of sperm obtained from refrigerated epididymis and fresh ejaculation of buck under 2 culture media, viz. modified synthetic oviductal fluid (mSOF) and research vitro cleave-blastocyst (RVCL-BLAST) media. In the process, the cauda epididymis of slaughtered buck were immediately collected, pre-processed and refrigerated (4°C) for 24 h. The oocytes recovered from slaughter house ovaries were *in vitro* matured and fertilized with both semen sources. On analysis of embryonic development, ejaculated group showed significantly higher blastocyst rate than epididymal sperm, however, initial cleavage rates of epididymal group was significantly higher than ejaculated semen, irrespective of culture media. Among the culture media, RVCL-BLAST media was significantly efficient than mSOF in terms of cleavage and blastocysts, irrespective of gametes sources. Despite of lower outcome of blastocyst, cold stored epididymal sperm may be considered as an alternative, potent and viable source of gametes for assisted reproduction in case of endangered or other livestock, if immediate facilities for sperm processing are not available.

**Key words:** Culture medium, Epididymis, Goat, *In vitro* fertilization, Semen

The efficient use of epididymal sperm could serve as an indispensable resource in propagation and conservation of elite livestock as well as endangered species (Santiago-Moreno *et al.* 2006) and pets (Leibo and Songsasen 2002). In the epididymis of slaughtered animals, the sperm remains alive for 24 to 48 h with fertilizing capacity (Dong *et al.* 2008). Artificial insemination (Santiago-Moreno *et al.* 2006), *in vitro* fertilization (Blash *et al.* 2004) and intracytoplasmic sperm injection (Jimenez-Macedo *et al.* 2005) gave satisfactory results with epididymal sperm. Sperm undergoes many biological processes during their trek to fertilize an oocyte. These processes include intracellular signaling, binding of receptors and an influx and efflux of ions through ion channels (Dragileva *et al.* 1999, Newton *et al.* 2010). Ejaculated and epididymal sperm are different in terms of exposure to seminal plasma. Epididymal sperm (fresh/cryopreserved) of many species are used for artificial insemination in bull (Guerrero *et al.* 2008), stallion (Monteiro *et al.* 2011), goat (Blash *et al.* 2004) etc. Embryo production by *in vitro* fertilization using epididymal sperm was accomplished in cow (Pavlok *et al.* 1988), swine (Rath and Niemann 1977), sheep (Wani *et al.*

2012), camel (Badr and Abdel-Malak 2010) etc. There is scope of more research to explore the chances of propagation and conservation of genetically valuable male animals even after their loss. The optimum use of epididymal sperm for an extended period would be an important step in case of non-availability/limited facility of immediate cryopreservation or assisted reproduction set up. Thus, the present study was designed to optimize the technique for long term use of gametes of buck cauda epididymis for IVF. This study represents a comparative *in vitro* fertilizing potential of sperm derived by ejaculated and refrigerated epididymis by assessment of embryo production rates under different culture media.

### MATERIALS AND METHODS

**Collection of ovaries and cauda epididymis:** Goat ovaries and cauda epididymes were collected from local abattoir in sterile warm normal saline (37°C) and transported to the laboratory within 3 h of slaughter. The ovaries and cauda epididymis were washed thrice with sterile normal saline and twice with Dulbecco's phosphate buffer saline (DPBS), followed by brief exposure of 70% ethanol for 10–15 sec and finally rinsed with DPBS. The epididymis was fully dipped in DPBS and stored at refrigerator temperature (4°C) for 24 h.

**Oocytes recovery and *in vitro* maturation:** Cumulus oocytes complexes (COC's) were aspirated from 4–8 mm

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sized follicles. The COC's having  $\geq 2$  cumulus layers and homogenous ooplasm were kept in groups of 20–25 numbers into 50  $\mu$ l drops of maturation medium including TCM-199, 7.5% (v/v) fetal bovine serum, 2.5 unit/ml follicle stimulating hormone, 2.5 unit/ml leuteinizing hormone, 1  $\mu$ g/ml estradiol, 0.8 mM/ml sodium pyruvate and 50  $\mu$ g/ml gentamicin under humidified atmosphere of incubator having 5% CO<sub>2</sub> at 38.5°C for 27 h. The maturation of oocytes was assessed on the basis of cumulus expansion and oocytes having good cumulus expansion were taken for IVF experiments.

**In vitro fertilization:** The refrigerated epididymis was thawed by gradual exposing to lukewarm DPBS for 45–60 sec and at 38°C for 3–5 min. The sperm were harvested by multiple incisions on epididymis and collected in a 15 ml centrifuge tube with 10 ml DPBS. The fresh semen was collected twice a week from a genetically proven buck by artificial vagina method. For both groups, immediately after collection, the volume, color, concentration, morphology, initial and gross motility of sperm were assessed. The sperm suspension was centrifuged at 1,000 rpm for 8 min. The supernatant was removed and remaining pellet (1 ml) was equally subdivided (0.2 ml) into 5 tubes of 2 ml. In each tube, 1 ml Bracket and Oliphant (BO) medium was added, mixed well and incubated at 38.5°C for 1 h in humidified atmosphere. From upper layer, 0.6 ml of sperm suspension from each tube was pooled into a 15 ml centrifuged tube. The final volume of the suspension was made up to 10 ml by adding equilibrated 10 ml BO medium. The suspension was centrifuged at 1,000 rpm for 8 min. The supernatant was removed and pellet was washed with 10 ml BO medium supplemented with 0.3% bovine serum albumin (BSA) and subsequently with 10 ml fertilization media (BO medium containing 0.6% BSA and 50  $\mu$ g/ml heparin). Finally, the sperm pellet was diluted with 2 ml of fertilization medium and transferred into a microcentrifuge tube held at 45° angle at 38.5°C for 45 min. In the mean time, *in vitro* matured oocytes (Fig. 1A) were transferred into 50  $\mu$ l microdrops of pre-equilibrated fertilization medium for 30–45 min. From the top layer of sperm samples, aliquots of sperm suspension were added into the droplets to achieve a final concentration of 10<sup>6</sup> sperm/ml. The oocytes were allowed to co-incubate with sperm for 18 h under humidified atmosphere of 5% CO<sub>2</sub> at 38.5°C.

**Embryo culture:** For both the groups, after 18 h of co-incubation, the oocytes were denuded with slight agitation by fine bored pasteur pipette. The presumptive zygotes obtained from both groups were cultured in 2 different types of culture media, viz. (1) modified SOF (mSOF) supplemented with 0.2 mM glucose and 0.8% fatty acid free bovine serum albumin (FAF-BSA) for the first 48 h followed by 1.5 mM glucose and 10% fetal bovine serum up to the 8<sup>th</sup> d, and (2) RVCL-BLAST- Research vitro cleave medium with 1% FAF-BSA for first 3 d followed by Blastocyst medium with 1% BSA up to the 8<sup>th</sup> d.

**Statistical analysis:** In the present study, 40 IVF experimental trials were conducted using 20 tests for each

of the fresh ejaculated and cold stored epididymal sperm groups. Within both groups, 10 replicates were done under each of the 2 media, viz. mSOF and RVCL-BLAST. The comparative embryonic developmental rates derived by 2 sperm sources under different media were analyzed by factorial analysis (Snedecor and Cochran 1994).

## RESULTS AND DISCUSSION

**In vitro maturation of oocytes:** Oocytes (3,210) were aspirated from 1,012 goat ovaries, in which 1,830 ( $\geq 2$  cumulus layers) were set for *in vitro* maturation. Out of 1,830 oocytes, 1,375 (75.14%) exhibiting good cumulus expansion was subsequently used for IVF (Fig 1A).

**Comparative assessment of fertilizing capacity of gametes sourced from cold stored epididymis and fresh ejaculation under two different culture media:** To avoid buck to buck variation, only one buck was used to collect fresh ejaculated semen in all experiments. In the present study, RVCL-BLAST media showed significantly higher ( $P \leq 0.05$ ) development of embryos as compared to mSOF, irrespective of the gametes source. In fresh ejaculated group, the RVCL-BLAST showed significantly higher ( $P \leq 0.05$ ) developmental rates of 2–4 cells (63.12 $\pm$ 1.01% vs 44.46 $\pm$ 0.65%), 8–16 cells (58.02 $\pm$ 0.63% vs 40.49 $\pm$ 0.83%), morulae (38.51 $\pm$ 0.66% vs 20.59 $\pm$ 0.42%), blastocysts (31.64 $\pm$ 0.94% vs 9.37 $\pm$ 1.0%) as compared to mSOF. Similarly in epididymal group, 2–4 cells (69.29 $\pm$ 0.63% vs 57.81 $\pm$ 0.48%), 8–16 cells (64.38 $\pm$ 0.81% vs 53.59 $\pm$ 0.7%), morulae (40.91 $\pm$ 0.61% vs 22.19 $\pm$ 0.84%) and blastocysts (28.91 $\pm$ 0.21% vs 7.78 $\pm$ 0.56%) formation rates were significantly higher ( $P \leq 0.05$ ) in RVCL-BLAST than mSOF medium (Figs 1B, C, D). Our results showed that the initial cleavage rates under epididymal group were significantly higher ( $P \leq 0.05$ ) than ejaculated gametes, however, the blastocyst formation rate by ejaculated sperm were significantly higher ( $P \leq 0.05$ ) than epididymal gamete, irrespective of culture medium (Table 1).

Genetically superior males have potential of improving

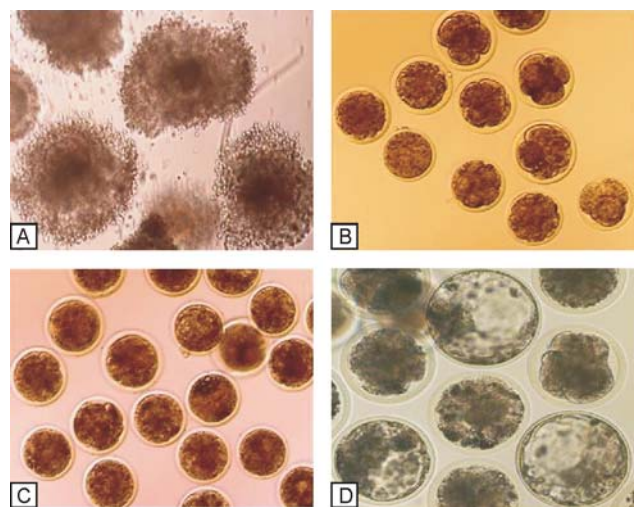


Fig. 1. Developmental stages of IVF goat embryos: (A) IVM oocytes, (B) cleavage, (C) morula, and (D) blastocysts.

Table 1. Developmental rates of IVF goat embryos produced by fresh ejaculated and cold stored

Sources of gametes	Culture medium	Embryonic developmental stages (mean±SE)				
		No. of oocyte	2–4 Cells(%)	8–16 Cells (%)	Morula (%)	Blastocyst (%)
Fresh ejaculation	mSOF	358	(50.07±1.99) <sup>d</sup>	(45.53±2.02) <sup>d</sup>	(24.98±0.86) <sup>b</sup>	(13.51±0.62) <sup>c</sup>
	RVCL-BLAST	343	(64.018±0.92) <sup>b</sup>	(58.85±0.67) <sup>b</sup>	(40.49±0.85) <sup>a</sup>	(31.76±0.55) <sup>a</sup>
Cold stored epididymis	mSOF	336	(59.77±1.04) <sup>c</sup>	(54.70±1.35) <sup>c</sup>	(24.58±1.17) <sup>b</sup>	(10.03±0.89) <sup>d</sup>
	RVCL-BLAST	338	(69.27±0.40) <sup>a</sup>	(63.62±0.52) <sup>a</sup>	(41.36±0.66) <sup>a</sup>	(29.01±0.17) <sup>b</sup>

the productivity of livestock species through quick dissemination of the superior germplasm in the population with the help of assisted reproduction technology. The injury/death of superior male ultimately affects the productivity. In such cases, epididymal sperm provides a last chance to conserve the germplasm of superior male, so that the sperm can be utilized in efficient manner for improving the production of livestock species.

Ball *et al.* (1983) reported for the first time the use of bovine epididymal sperm in IVF. Pavlok *et al.* (1988) reported a fertilization rate of 72% for epididymal and 54% for ejaculated bull sperm in IVF. These reports established the fresh epididymal sperm as viable source of sperm for fertilization *in vitro*. The birth of live young mice following the storage of epididymal sperm within the epididymis up to 24 h at 22°C before collection allows for the possibility to collect epididymal sperm from other animals that suddenly die (Songsasen *et al.* 1988).

In our study, IVF goat embryos were produced using the sperm stored within the epididymis at 4°C for 24 h and compared with the embryos derived by fresh ejaculated semen under 2 different culture media. Although epididymal group showed significantly higher cleavage rates but, blastocysts were significantly lowered as compared to fresh ejaculated group, irrespective of media. In similar studies, Song *et al.* (1988) used fresh and cryopreserved epididymal goat sperm to fertilize oocytes *in vitro* which resulted into fertilization rates of 51 and 57%, respectively. Blash *et al.* (2000) reported similar cleavage and blastocyst developmental rates following IVF using cryopreserved ejaculated (37 and 4%) and epididymal (40 and 6%) sperm. Al-Timimi (2013) reported 87, 75 and 67% fertilization rate on IVF by sperm collected from epididymis of local Iraqi buck in TCM, TALP and MEM media, respectively. Although the above studies used epididymal gametes for IVF, but the present study seems to be first report of using sperm of cold stored epididymis for production of IVF goat embryos

Since, exposure to the accessory sex gland fluids constitutes the principal difference between epididymal and ejaculated sperm therefore *in vitro* fertilizing ability of ejaculated and epididymal sperm may be different due to the lack of exposure to seminal plasma. Pavlok *et al.* (1988) reported that although fresh epididymal bull sperm showed higher fertilization rate also had an increased incidence of fertilization anomalies (32.6%) as compared to fresh ejaculated sperm (5.4%). The above findings might be one of the reasons in our study, for lower blastocyst production

rate in epididymal sperm despite of higher initial cleavage as compared to fresh ejaculated sperm. The findings of Soler *et al.* (2005) in Iberian Red Deer, Yu and Leibo (2002) in canines and Iranpour and Valojerdi (2003) in mice, suggested that functional sperm in terms of motility and membrane integrity could be recovered from epididymis stored at refrigerated temperature after slaughter. Likewise, Kishikawa *et al.* (1999), reported increasing incidence of sperm chromosome abnormality in mice with increasing period of storage after death but at least 20–30% of spermatozoa were having normal chromosomes and were potentially fertile even after 15 days of storage within cadavers or epididymis at refrigerated temperature.

Furthermore, the developmental competency of *in vitro* produced embryos depends on various important factors culture media being one of them. In this study, the development of embryos fertilized *in vitro* was significantly higher in RVCL-BLAST medium as compared to mSOF media, irrespective of gamete sources. Although RVCL medium was used for *in vitro* embryo culture by researchers (Shah *et al.* 2008, Jena *et al.* 2012) but, RVCL-BLAST media was first reported for parthenogenetic goat embryo culture by our laboratory (Kumar *et al.* 2014). The present study also supported the previous findings in terms of better embryonic development of IVF embryos under RVCL and Blastocyst media as compared to mSOF.

In this study, spermatozoa within cold stored epididymis were successfully used for *in vitro* fertilization in goat. While comparing the sperm sources, ejaculated gametes produced higher rates of blastocyst as compared to cold stored epididymal sperm. Also, it was observed that RVCL-BLAST induced better embryonic development rate as compared to mSOF medium irrespective of sperm sources. Despite of lower outcome, cold stored epididymal sperm may be considered as an alternative, potent and viable source of gametes for assisted reproduction in case of endangered or other livestock, if immediate ART facilities are not available in the field conditions.

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