



Comparison of Density Gradient and Swim-up Methods on Sperm Quality and *in vitro* Embryo Development in Sahiwal Cows

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ABSTRACT

Background: Low blastocyst yield and conception rate following *in vitro* fertilization and embryo transfer is one of the major limitations. To address this issue, semen separation methods are analyzed.

Methods: The study was conducted on sahiwal donor cows and non-descript recipient cows. Frozen semen of a Sahiwal bull was used for *in vitro* fertilization purpose following its preparation either by density gradient method (Group-I, n=10) or by swim-up method (Group-II, n=10).

Result: After semen separation by Group-I and Group-II, semen motility was noted to be $87.50 \pm 1.54\%$ and $69.50 \pm 1.35\%$, respectively and viability was $80.70 \pm 1.43\%$ and $57.10 \pm 2.20\%$, respectively. The plasma membrane intact spermatozoa was observed to be $83.80 \pm 1.47\%$ and $61.70 \pm 1.95\%$, respectively and DNA fragmentation index was noted to be $5.20 \pm 0.84\%$ and $7.80 \pm 0.59\%$, respectively following semen separation in Group-I and Group-II. From the ovum pick-up sessions, total 161 and 167 oocytes were aspirated in Group-I and Group-II. These oocytes were *in vitro* matured and *in vitro* fertilized using the semen sample prepared by Group-I and Group-II. On day 7th of the *in vitro* culture, blastocysts conversion rate of 98 (60.87%) and 46 (27.54%) was noted Group-I and Group-II of semen separation. Following transfer of *in vitro* fertilized embryos by the semen sample prepared by Group-I and Group-II of semen separation, average conception rate of 27.55% (27 pregnancies) and 15.22% (7 pregnancies) had been reported.

Key words: Blastocyst, Conception rate, Density gradient method, Swim-up.

INTRODUCTION

Dairy cow is essential for reducing rural poverty and it is the backbone of Indian dairy industry. India is the world's highest milk producer country contributing over 25% of the world's milk production. One of the important objectives was Conservation and multiplication of superior germplasm in Indigenous cattle by OPU-IVF technology and standardization of IVF technique (Deshpande *et al.*, 2023). Comparatively, Indigenous breeds possess superior thermo-tolerance and the ability to maintain normal biological functions under adverse environmental conditions than exotic breeds (Srivastava *et al.*, 2019). *In vitro* fertilization (IVF) with Embryo Transfer (ET) technology has been introduced to conserve the cow breeds by increasing their reproductive efficiency (Nandi *et al.*, 2002), enhance the milk production and simultaneously ensure rapid multiplication of superior germplasm. Sire contributes to 50% of the nuclear genetic makeup but essentially 0% of the mitochondrial genetic makeup in an offspring. Sire's nuclear genome must be compatible with the dam's inherited mtDNA for optimal metabolic health. The successful outcome of IVF was depended mainly on gametes quality. Sperm separation methods improve the sperm quality with higher rate of progressive motility and morphologically normal spermatozoa. Such selection of spermatozoa separates motile sperm from nonmotile,

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removes seminal plasma, cryo-protective and infectious agents, other background materials and debris (Henkel *et al.*, 2003) and initiates the capacitation of sperm (Centola *et al.*, 1998).

The less selective environment of *in vitro* production compared to the female reproductive tract allows for the

survival and development of lower-quality bovine embryos that would typically be eliminated by natural maternal filters. This absence of rigorous biological control in the oviduct and uterus often leads to embryos with reduced viability, altered gene expression and lower pregnancy rates (Van Soom *et al.*, 2007). Both fertilization failure and failure in subsequent *in vitro* bovine embryonic development are of seminal origin (Saacke *et al.*, 2000). The *in vitro* blastocyst and pregnancy rates remain considerably lower than those obtained for embryos produced *in vivo* (Hansen *et al.*, 2006). The present study was designed to compare the efficiency of sperm separation methods *i.e.*, Density gradient (Percoll) and Swim-up methods in terms of evaluating sperm microscopic parameters *i.e.*, motility, viability, plasma membrane integrity and DNA integrity and subsequent developmental rate of *in vitro* embryos.

MATERIALS AND METHODS

The present study was carried out at the *in vitro* Fertilization-Embryo Transfer Technology Laboratory, Department of Animal Reproduction, Gynaecology and Obstetrics, Nagpur Veterinary College, Nagpur from October 2024 to January 2025.

A total of 10 high-yielder Sahiwal cows with annual lactation yield of >3500 litre, BCS of more than 3 (scale 1-5) and free from any infectious diseases were selected as Donor cows. Ovum pick-up (OPU) was performed in FSH stimulated donor cows with the help of transvaginal ultrasound probe having oocyte aspiration assembly along with aspiration pump (WTA, Brazil). Following epidural anaesthesia, the transducer (Exago, IMV, France) was inserted into the vaginal canal and fixed in the vaginal fornix and the ovary was positioned against the head of the transducer per rectally. All visible follicles were aspirated by using a portable scanner that had a 5.0 MHz convex transvaginal array transducer and a stainless-steel needle guide (18 G; WTA, Brazil). The aspiration needle was attached to aspiration equipment and a vacuum system at negative pressure of 70 mm Hg. A tubing system of 1.1 mm inner diameter, 120 cm long (WTA, Brazil) was connected to a 50 mL conical tube containing 15 mL of OPU media kept at 37°C.

After filtration through 75 µm filter (WTA, Brazil) and Euroflush media (IMV, France), the retrieved follicular content was examined under stereo zoom microscope. The collected oocytes of Grades A, B and C in the petri dish were subjected to *in vitro* maturation for next 22-24 hours in benchtop incubator. The matured oocytes were washed and transferred to IVF medium (Vitrogen, Brazil). The concentration of spermatozoa in frozen straws was 20 million/ mL with initial motility (>70-80%). The semen straw of pedigree bull was thawed. The Density gradient and Swim-up semen separation methods were carried out as followed.

Density gradient method (Percoll method)

This method has been commonly used to clean out dead sperms in reproductive laboratory using only 2 different

density layers of solution (Promthep *et al.*, 2016). Semen undergone specific preparation steps-primarily thawing and often washing or dilution before being layered onto the density gradient.

Preparation of 10× concentrated salt solution (Buffer)

- Composition: 2.889 g NaCl, 0.238 g KCl, 0.116 g KH_2PO_4 , 0.112 g CaCl and 0.163 g HEPES Buffer dissolved in 50 mL distilled water.
- Alternatively, 10× Sperm-TALP or PBS (consisting of 1.37 M NaCl and 27 mM KCl) can be used. The 10× solution was filtered and autoclaved (121°C for 20 min) and stored at 4°C.

To make the 100% Isotonic Percoll (100% SIP), mix Percoll with the 10× buffer at a 9:1 ratio. The final density should be ~1.123 g/mL (in saline) and osmolality was adjusted to 280-320 mOsm/kg.

The 100% SIP is diluted using a standard sperm washing medium, such as Hepes-TALP or BO medium.

- 90% Percoll Solution (Bottom Layer): Mix 9 parts of 100% Isotonic Percoll (SIP) with 1 part of washing medium (e.g., 9 mL SIP + 1 mL Hepes-TALP).
- 45% Percoll Solution (Top Layer): Mix 1 part of the 90% Percoll solution with 1 part of washing medium (e.g., 1 mL 90% Percoll + 1 mL Hepes-TALP).

Frozen thawed semen was layered on the gradient prepared using upper and lower isolates of percoll in a 2.00 mL microcentrifuge tube and the mixture was centrifuged at 4700 rpm for 6 minutes. After discarding the supernatant, the pellet was aspirated and transferred to the pre incubated IVF medium and centrifuged again at 1700 rpm for 3 minutes. The sperm pellet was mixed thoroughly and the washed sperms were inoculated in the IVF droplets containing the matured oocytes. Density gradient centrifugation was also used for sexing spermatozoa because of the additional DNA content and volume of X-bearing sperm head (Bhat *et al.*, 2020).

Swim-up method

2 ml of diluted semen was taken in Falcon tube and centrifuged at 500 G for 5 minutes. The sperm pellet was resuspended in 1 mL TCM-199 with antibiotics which was then placed at the bottom of four falcon tubes (0.25 mL each) and 1 mL of TCM-199 medium was layered in each Falcon tube above the suspension. Each tube was then incubated at 38.5°C for 1 hour for swim-up. Further, the top 0.25 mL layer from each of the four tubes was removed and pooled together in one tube. The layer resuspended in 1 mL of TCM-199. To this 10 µg/mL heparin was added and placed in incubator. After semen separation by swim-up method, the petri plate containing the oocytes and sperm was incubated in a benchtop incubator at 38.5°C.

The microscopic semen parameters like Motility, Viability by Eosin-Nigrosin staining (Fig 1), Plasma membrane integrity by Hypo-osmotic swelling test (Fig 2) and DNA fragmentation index by acridine orange test (Fig 3) were studied post thaw and with the pellet obtained after density

gradient method and swim up method. After 18 hours, the presumptive zygotes were washed and the connected cumulus cells were removed using a 140 µm diameter

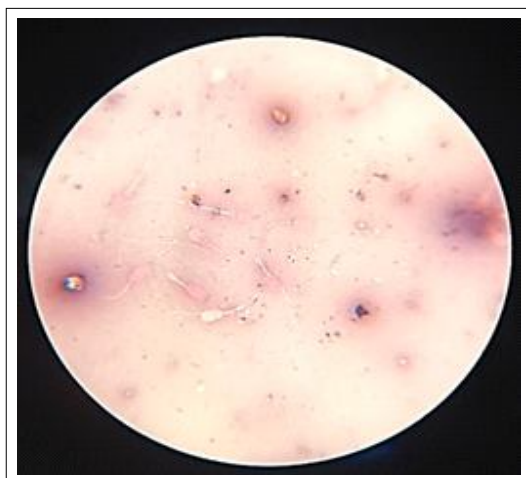


Fig 1: Eosin nigrosin staining.

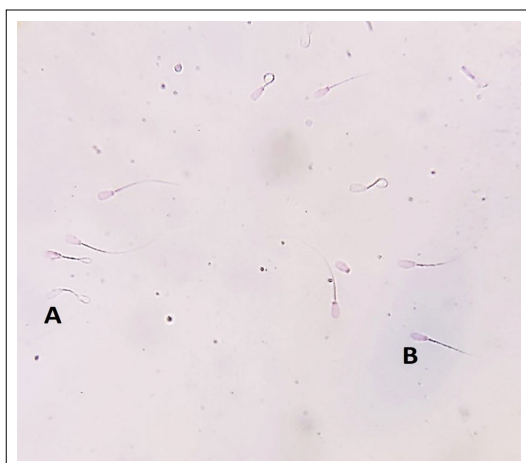


Fig 2: HOST reactive spermatozoa (A)
HOST non-reactive spermatozoa (B).

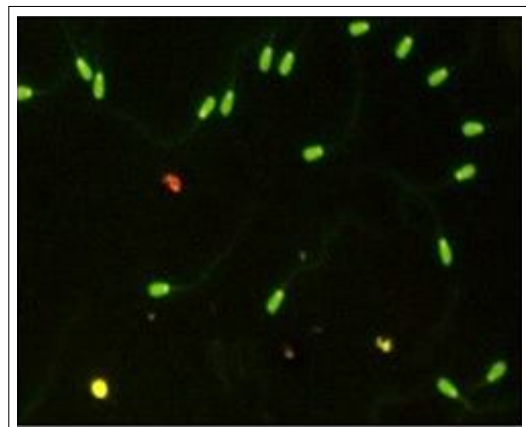


Fig 3: Acridine orange staining spermatozoa with green fluorescence-intact DNA, spermatozoa with orange fluorescence-damaged DNA.

glass pipette attached to stripper (Cook, Australia). The presumptive zygotes were transferred to the IVC medium (Vitrogen, Brazil) for the next seven days.

On 7th day of IVC, the blastocyst production rate following IVF with semen separated by density gradient and swim-up method was assessed accordingly. The embryo transfer was done in close to the tip of the ipsilateral horn to the side of corpus luteum in recipient cows. Pregnancy diagnosis was carried out transrectally using ultrasound machine (Z5 Vet, Mindray, China) at 45 days of embryo transfer in the recipients. Conception rate following transfer of blastocysts in both the treatment groups was compared accordingly. The collected data was statistically assessed per the WASP 2.00 ICAR, Goa.

RESULTS AND DISCUSSION

As shown in Table 1, ten ovum pick up sessions were conducted each in Group-I and Group-II methods aspirating total 328 oocytes. These oocytes were *in vitro* matured and *in vitro* fertilized using the semen sample prepared by Group-I and Group-II methods. Subsequently, different microscopic characteristics of frozen thawed semen were studied.

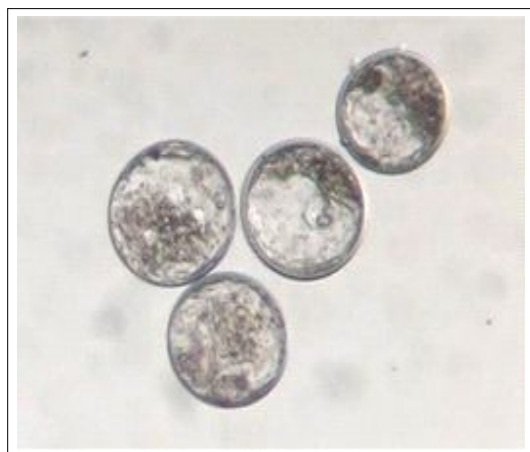
The present findings of semen motility following separation by density gradient method and swim-up method are in accordance with the earlier findings of García-López *et al.* (1996) in ram, Palomo *et al.* (1999) in bull, Rho *et al.* (2001), Trentalance *et al.* (2002) and Jamil *et al.* (2007) who stated that percoll gradient method is the best method for separation of highly motile spermatozoa from the poor-quality semen post thaw. Viability of semen showed significantly higher sperm viability following semen separation by density gradient method than swim-up method. Similar findings are reported by Valcárcel *et al.* (1996); Somfai *et al.* (2002); Trentalance *et al.* (2002) and Jamil *et al.* (2007). Significantly higher plasma membrane intact spermatozoa is observed following semen separation by density gradient method than the swim-up method. Valcárcel *et al.* (1996), Samardzija *et al.* (2006) and Jamil *et al.* (2007) also recorded the similar findings and hence are in agreement with the findings of present study.

On day 7th of the *in vitro* culture, the overall blastocysts conversion rate of 98 (60.87%) and 46 (27.54%) was noted in Group-I and Group-II methods. In the present study, significantly higher blastocysts conversion rate was recorded in density gradient method (Group-I) than in swim up method of semen separation. Seidel *et al.* (1995) and Larocca *et al.* (1997) also recorded that density gradient method yields better blastocysts yield than swim-up method. Rho *et al.* (1998); Rho *et al.* (2001) and Matas *et al.* (2003) noted that density gradient method separated the highly motile population of spermatozoa, thus yields higher blastocysts conversion rate as compared to other methods of semen separation.

Following transfer of *in vitro* fertilized embryos (Fig 4) by the semen sample prepared by Density gradient (Group-I)

Table 1: Average values of different microscopic semen characteristics and blastocysts yield, conception rate following IVF with semen sample prepared by Group-I and Group-II.

Parameters	Group-I (Density gradient method)	Group-II (Swim-up method)
Number of OPUs	10	10
Number of oocytes aspirated	161	167
Post thaw motility (%)	74.00±2.33	70.50±2.41
Motility after semen preparation (%)	87.50±1.54	69.50±1.38
Post thaw viability (%)	57.90±2.35	52.60±2.95
Viability after semen preparation (%)	80.70±1.43	57.10±2.20
Post thaw plasma membrane integrity (%)	63.30±2.85	60.40±2.90
Plasma membrane integrity after semen preparation (%)	83.80±1.47	61.70±1.95
Post thaw DNA fragmentation index (%)	27.00±3.47	23.90±1.95
DNA fragmentation index after semen preparation (%)	5.20±0.84	7.80±0.59
Number of blastocysts produced	98 (60.87%)	46 (27.54%)
Conception rate	27 (27.55%)	7 (15.22%)

**Fig 4:** Embryos produced after IVF.

and Swim-up method (Group-II) of semen separation, average conception rate of 27.55% (27 pregnancies) and 15.22% (7 pregnancies) had been reported in the present study. Larocca *et al.* (1997); Alomar *et al.* (2006) and Cesari *et al.* (2006) also reported improved conception rate following use of semen separated by density gradient method which is in accordance with the findings of present study.

CONCLUSION

Following semen separation by Density gradient method (Group - I), significantly higher semen motility, viability, plasma membrane integrity and lower DNA fragmentation index was observed than in semen separation by Swim-up method (Group - II). Also, significantly higher blastocyst yield and conception rate had been reported with Density gradient (Group - I) than in Swim-up (Group - II) method of semen separation.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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