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# Effect of Dilution and Sperm Concentration on Post Thaw Semen Quality in Barbari Buck

**Mukul Anand[1*], Sarvajeet Yadav[1], Alok Kumar[2], Shalini Vaswani[3] and Pankaj Shukla[4]**

*[1]Department of Veterinary Physiology, College of Veterinary Sciences and Animal Husbandry, U. P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, Uttar Pradesh, INDIA*
*[2]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Sciences and Animal Husbandry, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura, Uttar Pradesh, INDIA*
*[3]Department of Animal Nutrition, College of Veterinary Sciences and Animal Husbandry, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura, Uttar Pradesh, INDIA*
*[4]Department of Poultry Science, College of Veterinary Sciences and Animal Husbandry, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan,Mathura, Uttar Pradesh, INDIA*

*Corresponding author: M Anand; E mail: drmukulanandvet@gmail.com*

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**ABSTRACT**

Deleterious interaction between seminal plasma proteins and egg yolk has been well documented in buck semen. Reduced lethal contact between seminal proteins and egg yolk can be a prerequisite for improved post thaw semen quality. Sperm concentration that determines seminal plasma volume in extender regulates quantum of lethal proteins and intensity of lethal interaction in diluted goat semen. Therefore, experiment was designed to evaluate the effect of dilution and sperm concentration in diluted buck semen utilized for cryopreservation and artificial insemination. Semen from four healthy Barbari bucks was collected and pooled. Pooled semen was equally divided and diluted with TRIS based extender containing 6% glycerol and 20% egg yolk at four different sperm concentration *viz.* 100 million /ml, 200 million /ml, 300 million /ml and 400 million /ml and cryopreserved. Frozen thawed semen was evaluated for progressive motility, live percent, hypo-osmotic swelling test and motility parameters using computer assisted semen analyzer (CASA). A significantly ($p < 0.01$) higher value for live percent, progressive motility, HOST percent, VCL (µm/sec), VAP (µm/sec), VSL (µm/sec), BCF (hz) and DNC ($\mu m^2$/sec) were observed in semen diluted to 200-million/ml of sperm concentration. The results depict a better counterbalance between the different factor influencing the viability, membrane integrity, path velocity and motion characters in semen diluted to 200-million/ml of sperms as compared to other sperm concentration in diluted semen. In conclusion, 200-million/ml sperm can be preferred concentration for semen dilution and cryopreservation that result in better post thaw semen quality in buck semen.

**Keywords:** Buck, Semen, Cryopreservation, Sperm motility, CASA.

Semen dilution and cryopreservation are two techniques that provide opportunity to utilize single ejaculate as multiple insemination doses. Insemination doses from a freshly collected ejaculate largely depends upon precise assessment of viable and motile sperm in unit volume of semen. Based upon these parameters, sperm concentration and dilution factor has been well established in most of farm species. But in goats, seminal proteins react with egg yolk in extender to form compounds (lysophophatidylcholine) lethal to sperm (Leboeuf *et al.,* 2000), thus limits a standard protocol for semen dilution. The quantum of seminal reactive proteins and egg yolk level in extender determines the intensity of lethal interactive losses reflected through sperm damage and poor semen quality.

Majority of work to reduce interactive losses and improve post thaw semen quality are associated with use of variable egg yolk level in extender. Bispo *et al.* (2011) reported that egg yolk at 3% level in extender is better when compared

with 20% egg yolk in extender at a concentration of 400 million sperm/ml in goats, while a better post thaw semen quality was observed at 20% egg yolk level in extender at concentration of 100 million sperm/ml (Anand *et al*., 2016). Similarly, variable results have been observed at particular egg yolk level with different sperm concentration in diluted semen (Sundararaman *et al*., 2008; Ustuner *et al*., 2009; Cebi *et al*., 2015). These finding indicate role of sperm concentration in determining post thaw semen quality in diluted goat semen. However, no standard sperm concentration at particular egg yolk level been established and a précised dilution factor for ejaculate in goats is still lacking (Fernandez-Santos *et al*., 2006). So, the present experiment was designed to evaluate effect of dilution and sperm concentration on post thaw semen quality in Barbari buck.

## MATERIALS AND METHODS

The experiment was conducted in north western semi-arid zone of India with latitude of 27.49 °N and longitude of 77.67 °E with an elevation of 177 m from mean sea level during month of September to November (autumn season). Four healthy Barbari bucks of similar age (2.0–2.5 years) and weight (range 35-40 kg) maintained at Departmental goat farm were selected as semen donor. The semen was collected biweekly from each buck using artificial vagina. The collected semen from four bucks was immediately placed in insulated thermo-flask maintained at 37°C and taken to semen analysis laboratory. The samples with >3.5 mass motility, > 85% live sperm, >75% progressive motile sperms and < 10% sperm abnormality were selected and pooled to minimize individual variation and evaluated for sperm concentration and viability. The samples were then divided into equal 4 aliquot and diluted as per the treatment using TRIS based extender with 6% glycerol and 20% egg yolk. Spermatozoa concentration was maintained at 100 million/ml (Group -1), 200 million/ml (Group -2), 300 million/ml (Group -3) and 400 million/ml (Group -4) and subjected to cryopreservation. The straws were stored in liquid nitrogen ($LN_2$) till analysis. The frozen thawed semen was evaluated for progressive motility (%), Live percent (Hancock, 1952), Hypo-Osmotic Swelling Test (Jeyendran *et al.* 1984) and CASA Analysis (Biovis CASA 2000, Version 4.6, India). Settings of CASA system (Biovis CASA 2000, Version 4.6, India) designed using algorithm based on size, shape, detection of sperm head and classes for motile, immotile, rapid, slow and non-progressive were as follow: Frames/s - 60, number of frames acquired - 61, max velocity (for tracking): V (µm/s) - 150 motility min, curvilinear velocity (VCL) (µm/s) - >25 motility min, average path velocity (µm/s) - >10 motility min, straight-line velocity:(µm/s) - >1 min, track length (% of frames) - 51, aspect - 0-99,999, area - 2-20, axis (major) - 4-20, axis (minor) - 2-10, compactness - 0-50, perimeter ratio - 0-99,999, minimum cell size on major axis - 20, minimum cell size on minor axis – 10, magnification - ×10 phase, calibration × (pixels/unit) - 1.905 µ, Y (pixels/unit) - 1.905 µ, size of image - 1280 × 960 pixels. For CASA analysis, a 4 µl of diluted semen sample ($50 \times 10^6$ spermatozoa per ml) was loaded in Makler sperm counting chamber with surface graticule of 100 × 0.01 sqmm (Sperm processor, Welcomenagar, India) and a range of 3-6 fields were acquired for motility analysis. The following motility indicators were measured: VCL (average cell velocity along the actual path, µm/s), VAP (average path velocity of the smoothed cell path, µm/s), VSL (average straight velocity for the movement along a straight line from beginning to end, µm/s), LIN (of movement as a ratio VCL/VAP, %), STR (straight line character of movement as a ratio of VSL/VAP, %), WOB (degree of oscillation of the actual path of the sperm head in his relationship with the VAP, BCF (average rate at which the actual sperm trajectory crosses the VAP, ALH (amplitude of lateral head displacement, µm), DNC (VCLxALH, $\mu m^2$ /s).

### Statistical analysis

The data recorded during the experiment were analyzed using Statistical Package for Social Science (SPSS® Version 20.0 for Windows®, SPSS Inc., Chicago, USA). The means were compared using Analysis of Variance, Duncan's multiple range tests and presented as mean ± standard error (SE).

## RESULTS AND DISCUSSION

The effect of sperm concentration on viability, progressive motility and membrane integrity (HOST) in frozen thaw Barbari buck semen has been presented in Fig. 1. Significantly ($p<0.01$) higher value for live percent, progressive motility and HOST percent was observed at 200 million/ml concentration of spermatozoa, followed by 100 million/ml and then 300 million/ml and 400 million/ml. A

**Table 1:** Effect of different sperm concentration on path velocities and motion characters of sperm in frozen thawed semen

| Parameter<br>Group | VCL µm / sec | VAP µm / sec | VSL µm / sec | LIN % | STR % | WOB % | BCF hz | ALH µm | DNC µm²/sec |
|---|---|---|---|---|---|---|---|---|---|
| Group –I<br>(100 million/ml) | 82.40$^{b}$<br>±2.11 | 36.60$^{b}$<br>±1.57 | 26.00$^{b}$<br>±1.67 | 31.46<br>±2.17 | 68.20<br>±3.49 | 44.90<br>±2.66 | 15.92$^{a}$<br>±1.32 | 3.74<br>±0.35 | 278.46$^{b}$<br>±20.99 |
| Group –II<br>(200 million/ml) | 97.20$^{a}$<br>±1.43 | 40.60$^{a}$<br>±1.57 | 32.40$^{a}$<br>±2.48 | 31.78<br>±2.77 | 74.14<br>±3.20 | 41.28<br>±2.26 | 17.26$^{a}$<br>±0.49 | 4.56<br>±0.40 | 368.58$^{a}$<br>±15.39 |
| Group –III<br>(300 million/ml) | 79.00$^{b}$<br>±4.97 | 26.80$^{c}$<br>±0.86 | 20.20$^{bc}$<br>±2.06 | 27.90<br>±4.82 | 72.58<br>±7.48 | 36.76<br>±2.54 | 12.46$^{b}$<br>±0.70 | 4.02<br>±0.57 | 254.90$^{b}$<br>±43.46 |
| Group –IV<br>(400 million/ml) | 63.20$^{c}$<br>±2.03 | 24.6$^{c}$<br>±1.03 | 18.00$^{c}$<br>±1.45 | 27.16<br>±1.35 | 63.40<br>±1.44 | 37.96<br>±1.14 | 9.92$^{b}$<br>±1.45 | 3.14<br>±0.27 | 148.40$^{c}$<br>±18.09 |

Means with different superscript letters (a, b, c) differ significantly ($p<0.01$) within a row. VLC=Curvilinear velocity, VAP=Average path velocity, VSL=Straight line velocity, LIN=Linearity, STR=Straightness, WOB=Wobble, BCF=Beat cross frequency, ALH=Amplitudelateral head displacement, DNC=Dance.

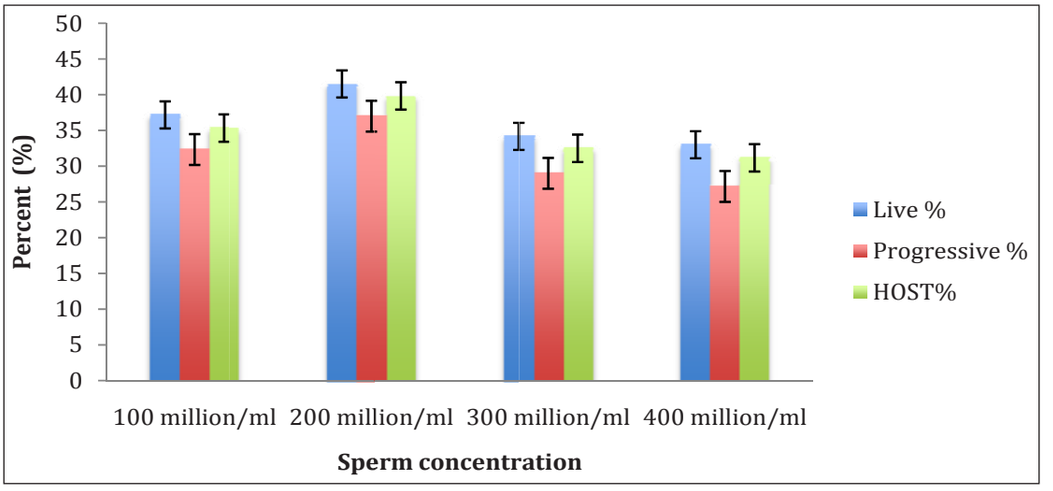


**Fig. 1:** Effect of different sperm concentration on physical seminal attributes in frozen thawed semen

significantly ($p<0.01$) high VCL (µm/sec), VAP (µm/sec), VSL (µm/sec) and BCF (hz) were observed at 200 million/ml as compared to other concentration, while Lin (%), Str (%), ALH (µm) and DNC (µm²/sec) were non-significantly higher at 200 million/ml concentration (Table 1). WOB was non-significantly ($p<0.05$) higher at 100 million/ml concentration as compared to sperm concentration. Seminal proteins provide nutrition and protection to sperm cells. Majority of proteins in seminal fluid are required for sperm metabolism, sperm function and their transport in the female reproductive tract. But few proteins in seminal plasma called as Goat Seminal Plasma (GSP) proteins are detrimental to sperm cells. These GSP proteins designated as GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa. They are structurally related to BSP family of proteins found in bull, boar and stallion (Villemure *et al.,* 2003). Similar to BSP, GSP proteins induce cholesterol and phospholipid removal from sperm membrane making them liable to cold shock and cryoinjuries. Further in goats, a seminal protein (SBUIII) and an enzyme secreted named egg yolk coagulating enzyme (EYCE) by the bulbourethral glands, may cause toxicity to sperm in egg

yolk or milk-based extenders (Purdy, 2006; Ferreira *et al*., 2014). The egg-yolk coagulating enzyme (EYCE) in seminal plasma (Roy, 1957) identified as phospholipase A (Iritani and Nishikawa, 1961) and SBUIII as a 55- 60 kDa glycoprotein lipase from the goat bulbourethral gland (BUSgp60) (Pellicer-Rubıo *et al*., 1997) act as a catalyst that hydrolysis egg yolk lecithin into fatty acid (Bispo *et al*., 2011) that is toxic to sperm (Pellicer-Rubıo *et al.,* 1997; Pellicer-Rubıo and Combarnous, 1998). These two factors regulate the post thaw semen quality in goats.

During the experiment, semen was diluted to variable sperm concentration for evaluating the dilution factor with least interactive losses for better post thaw semen quality. Semen dilution reduces the level of reactive proteins (GSP, SUBIII and Phospholipase $A_2$) adversely affecting semen quality during freeze thawing. Poor post thaw semen quality recorded with high sperm concentration and seminal plasma volume. The probable reason for lower values may be attributed to higher concentration of reactive seminal proteins at lower dilution that interacted with egg yolk in extender to form more lethal compound affecting sperm (Brinsko *et al*., 2000; Miro *et al*., 2009). The higher values observed for physical seminal attributes (% live sperm, % progressive motile sperm and HOST positive sperms) in 200 million/ml may be the result of a better counter balance between the lethal reactive looses, protective seminal proteins, extender constituents and egg yolk during the process. The cryopreservation incurs the losses on sperm plasma membrane (Watson, 2000). Any disturbance in the integrity of sperm membrane results in metabolic disturbance and affecting sperm kinematics. Curvelinear velocity (VCL, µm/sec), average path velocity (VAP, µm/sec) and straight line velocity (VSL, µm/sec) are path velocities indicative of path followed and total distance covered by sperm in unit time. They have positive correlation with sperm capacity to cross the reproductive barrier and reach fertilization site. The VCL (µm/sec) and VSL (µm/sec) are considered to be the most important characteristics to evaluate the kinetic characters that influence the fertilizing ability of spermatozoa (16). ALH (µm), VSL (µm/sec), VCL (µm/sec) and linearity (LIN, %) have also been reported to be correlated with fertility (Aitken *et al*., 1984; Barratt *et al*., 1993; Krause, 1995). Higher values of Beat Cross Frequency (BCF, hz) together with VCL (µm/sec) and VSL (µm/sec) indicate a correlation between motion parameters, morphology and functional integrity of sperm (Stachecki *et al*., 1993).

Cryopreservation of goat semen with egg-yolk extender at higher sperm concentration has been reported to have declining motility due to the presence of egg yolk coagulating enzyme (EYCE) in the seminal fluid (Ajadi *et al*., 2012). Contrary to this, removal of seminal plasma has been reported to maintains semen characters following freezing/thawing (Kozdrowski *et al*., 2007) indicating the role of sperm concentration in regulating postthaw semen quality at specific egg yolk level. Sperm velocity and path followed by sperm depend upon the mitochondrial energy production system and flagellar movements (Bezerra *et al*., 2011). Cryopreservation alter mitochondrial function, reduction of motility and failure of chromatin decondensation, during the process of cryopreservation which influence the viability and fertility of the sperm cells has also been reported by different authors (Watson PF, 2000; Chaveiro *et al*., 2006; Cooter *et al*., 2005; Wongtawan *et al*., 2006). In addition, lethal compounds that act like detergent on sperm plasma membrane stimulates the efflux of membrane constituents affecting the membrane permeability (Medeiros *et al*., 2002). This makes sperm more vulnerable to cryoinjuries and lipid peroxidation affecting its viability and motility.

Denaturation and efflux of flagellar proteins disturb the glycolytic ATP production system in the principal piece of the flagellum reflected through altered path velocities and motion characteristics of sperm cell (Miki *et al*., 2004; Cao *et al*., 2006). During the experiment semen dilution at 200 million/ml spermatozoa with better capacity to maintain the membrane integrity and established better coordination between the energy production and its utilization by sperm cell recorded higher values for path velocities. Lower values observed at 100 million/ml might have resulted from higher dilution reducing the level of protective proteins and antioxidative enzymes while the reason for lower values at higher sperm concentration may be increase intensity of lethal interactive losses affecting sperm characters. In conclusion, semen diluted with extender (20% egg yolk) to concentration of 200 million spermatozoa per ml has better capacity to maintain sperm characters and path velocities of frozen thaw sperm cells. Variable sperm concentration has no effect on the motion characters indicative of similar path adopted by sperm cell during its progression. In conclusion, semen

dilution to 200 million spermatozoa/ ml establish a better counterbalance between different constituents influencing the sperm characters and can be a preferred concentration to be utilized for semen dilution and cryopreservation of buck semen.

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