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Cryopreservation and fertility of frozen thawed Chegu goat semen

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ABSTRACT

Goats are important livestock species of India. Chegu is a pashmina producing goat native to the cold arid region of Himachal Pradesh (H.P.), India. Semen cryopreservation from six Chegu elite males (aged 2.05±0.40 years; weighed 29.16±2.02 kg) was practiced using Tris Citrate Egg Yolk extender containing 10% EY and 6% Glycerol. Gross semen parameters includes volume (0.80.85±0.07 ml), color (Creamy white to yellowish), concentration (2238.5±231.0 x10^6 spermatozoa/ml) and mass motility (3.92±0.03).The significant changes (P<0.01) in post thaw seminal parameters (75.48±0.69 *v/s* 37.38±0.90; progressive motility), viability (75.79±0.95 *v/s* 48.25±1.78), morphological abnormalities (5.64±0.29 *v/s* 7.02±0.32) and HOST reactive spermatozoa (64.07±1.75 *v/s* 43.35±1.79) were observed in present study. Artificial insemination using frozen thawed semen having concentration (150 x10^6 spermatozoa/straw) from three different bucks was practiced in 40 synchronized goats with conception rate of 42.5 per cent. Non-significant variations amongst different bucks were observed with birth of 1.12 kids per doe and twinning rate of 11.8 per cent. It was concluded that semen cryopreservation along with artificial insemination can be practiced in Chegu goats to improve the population of this endangered species.

Key words: Artificial insemination, Chegu goat, Fertility, Semen, Seminal plasma.

INTRODUCTION

Goats are important livestock species of India. Chegu is pashmina yielding breed of goats found usually at altitude of more than 8000 feet above mean sea level in cold desert areas and produce valuable textile fibre pashmina hence called as "Pashmina goats" (Dogra and Thakur, 2010). The breed characterstics includes better adaptability to high altitude topography, ability to survive on scarce forage resources particularly during winters with good reproductive and disease resistance abilities (Thakur *et al.*, 2005a). Goats contributes towards income and employment generation, capital storage and improvement in household nutrition.

Chegu goat population has been reported to be 7000 in Himachal Pradesh (H.P.) to 10,700 overall (19th Livestock Census, 2012) and therefore have been put under "Endangered species" as evidenced by an ever declining population due to several socio-economic reasons and lack of developmental policies for breed conservation and improvement leading to loss of breed utility and marginilization of goat population (Dogra and Thakur, 2010). Persistence with the same males in a flock has caused inbreeding of varying effect.

Decreasing number of Chegu goats needs a quick addressal in a systematic manner. While some concrete policy to check the degradation of goats is enforced in H.P., it will be worthwhile to stock the semen from elite males of Chegu goats and further propagate it by utilizing artificial insemination using frozen thawed semen. So the present study was planned to cryopreserve the Chegu goat semen and further utilize it for artificial insemination.

MATERIALS AND METHODS

Selection of animals and screening for diseases: The study was conducted on apparently healthy Chegu bucks (n=6) aged 2.05±0.40 years (1.2-3.6 years), weighed 29.16±2.02 kg, (24-34 kg). These bucks were selected on basis of breeding history, breeding soundness evaluation and testicular diameters. All the bucks were ear tagged numbered (2407, 2408, 2409, 2412, 2415 and 2417), respectively. All the bucks were maintained under identical conditions and were screened for diseases, Brucellosis using RBPT, OIE guidelines (2008), Chlamydiosis using AGPT, Chahota *et al.*, (2015) to eliminate the possible transmission of infection.

Location of animals and period of investigation: All the bucks were maintained at University Livestock Farm of CSK Himachal Pradesh Krishi Vishvavidyalya, Palampur (32.6°N, 76.3°E, altitude 1290.8m) from September 2017 to December 2017.

Climatic conditions during period of investigation: Average light**:**dark hours (h) and temperature (°C) during the semen collection (September-December, 2017) in Chegu goats were 7.11±0.47:16.89±0.47, 21.94±0.18°C; 9.08± 0.25:14.92±0.025, 19.84±0.30°C, 7.07±0.41**:**16.93±0.41,

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14.79±0.34°C and 7.72±0.51:16.28±0.51, 13.12±0.43°C, respectively. The temperature (°C) and sunshine (h) for the day of semen collection were considered to be the average values gathered from the day of immediately preceding semen collection to the day of collection under consideration. For the first semen collection the values for temperature and sunshine were derived by considering the values for previous four days.

Animal housing, feeding and training for semen collection: The Chegu bucks were subjected to grazing (G) condition for five hours in a day (9:00 to 12:00 h and from 14:00 to 16:00 h). During the remaining period, the animals were housed under confined (C) conditions in a shed. The Chegu bucks were fed as per the standards of Indian Council of Agricultural Research (ICAR, 2013). All males had round the clock access to the clean drinking water under C condition. Bucks were supplemented extra concentrate ration @3-3.5% of body weight containing DCP 11.59 per cent and TDN 77 per cent, respectively. Training for semen collection was accomplished by 4-5 exposures of each buck to an estrus doe.

Semen collection and evaluation: A total of 100 ejaculates from six adult healthy Chegu bucks were collected twice weekly by artificial vagina (AV) maintained at 42-43°C using induced estrus females as teaser. Ejaculates were primarily evaluated for colour, volume and concentration (Caprine Photometer, IMV 1409®) followed by microscopic examination for mass motility. Ejaculates were judged by classifying the semen sample as suitable or unsuitable according to standard criterion (Kharche *et al.*, 2013; Rather *et al.* 2016). Absence of any gross abnormality in semen colour, mass motility of ≥3 and initial progressive motility of ≥70 per cent were considered as main criterion for selection or rejection of semen sample. Microscopic examination includes Progressive motility (Hafez and Hafez, 2000), Live and Dead (Hannock, 1951), Morphological abnormalities (Bloom, 1977) and HOST reactivity (Pant *et al*. 2002) which was done using (Nikon Eclipse Ni®, DIC microscope, Nikon India).

Seminal plasma removal and extension: The seminal plasma was removed as described by Nuti, (2007). The collected sperms were washed in Ringer's solution (1:10) to remove the seminal plasma by centrifuging the solution twice at 2500 rpm for 7 minutes. The semen pellet thus obtained after removal of Ringer's solution was extended with two equal fractions of TEY extender (TRIS 1.21 gms, Citric acid 0.685 gm, D-Fructose 0.5 gm, Benzyl Penicillin 1000 IU/ml, Streptomycin Sulphate 1mg/ml) along with 10% Egg Yolk and 6% Glycerol. Ultrapure water was added to make total volume upto 50 ml. TEY extender was finally added at a gap of 2-3 minutes to yield a final concentration 150 $x10^6$ spermatozoa per 0.25 ml straw, equivalent to 600 $x10^6$ spermatozoa/ml. pH of the buffer was adjusted to 6.7 to 6.9.

Filling and sealing of straws: Extended semen was filled in 0.25ml French mini straws (IMV Technologies, L'Aigle, Cedex, France), by aspiration using micropipette (Minitube, Germany) and subsequently sealed at free end with the help of polyvinyl alcohol (PVA) (IMV Technologies, L'Aigle, Cedex, France) powder.

Equilibration, vapour exposure, freezing and thawing: The straws were laid on a stainless steel rack and placed in cooling cabinet (Macro Scientific works Pvt. Ltd. India) for 4h to attain a temperature of 30°C to 4°C. After equilibration rack of straws from cooling cabinet was shifted into a styrofoam box having LN_2. The quantity of LN_2 in styrofoam box was so decided that its upper surface remained 4 cm below the rack. Straws were exposed to LN_2 vapours for 7 minutes. Finally the straws were immediately shifted into liquid nitrogen goblet of a cryocan (Cryogem) for storage. The inventory of semen storage was also maintained. Thawing of semen straws was done at 37°C for 30 secs (Sariozkan *et al.*, 2010) in a water bath.

Artificial insemination: A group of 2-5 years healthy Chegu goats (n=40) of proven fertility (had at least one kidding) were selected for A.I. All the selected animals were managed by individual shepherd of Thangkarma village, District Kinnaur H.P. India. Animals were subjected to grazing (G) conditions for 6-8 h in a day (7:00 to 11:00 h and from 2:00 to 6:00 h). During remaining period goats were rested in open spaces. Estrous cycles were synchronized with Cloprostenol sodium @187.5µg (Pragma®; Intas Pharmaceuticals Ltd., India), i/m approximately 60 h prior to expected AI. Does were observed twice daily for behavioural signs of estrus after 36h of $PGF_2\alpha$ injection. Teasing with a mature buck for 30 minutes was also used for estrus detection. A total of 48 Chegu does were synchronized but eight animals did not manifest estrus response and were therefore excluded from study. The cervico vaginal discharge (CVD) was aspirated by method similar to that used for cattle prior to AI except for AI sheath that was cut to half for purpose of aspiration with syringe and adaptor. Depending on the arborization pattern the does in estrus were classified as being in proper or improper estrus. A double straw intra-cervical insemination was carried out with 0.25 ml straw each containing 150 $x10^6$ sperms. Semen samples with ≥40 per cent post thaw progressive motility, from three bucks (tag numbers 2407, 2408 and 2417), were utilized for AI. Simultaneous to AI, all the animals were injected with Buserelin acetate @ 4 µg (Receptal®; MSD Animal health), i/m.

Pregnancy and kidding evaluation: Pregnancy diagnosis was ascertained by transrectal ultrasonography (Linear rectal probe, of 7.5 MHz frequency) done at 60 days post AI. Kidding records for the AI undertaken in present study were also obtained.

Statistical analysis: The data obtained were analysed using package R version 3.4.3. Paired sample t-test was used to see the significant difference between fresh and post thaw evaluation of quality seminal parameters with TEY extender containing 10 per cent EY and 6 per cent Glycerol. Pregnancy rates for different bucks were analyzed using Chi square test. Results were presented as mean ±SEM and differences were considered significant when P<0.05.

RESULTS AND DISCUSSION

Gross and microscopic seminal parameters in Chegu goats have been tabulate in Table 1 and 2, respectively. The average semen volume in Chegu bucks (Table 1) in present study was higher than West African Dwarf bucks 0.38–0.44 ml, Waidi *et al.,* (2007), Chegu bucks 0.47 ml, Thakur *et al.,*(2005b), Pashmina bucks 0.62 ml, Mohan *et al.,* (1980), Gaddi bucks 0.66±0.04 ml Sharma, (2018), lower than Blanca Andaluza bucks 0.87-0.97 ml, Gallego-Calvo *et al., (*2015), Blanca-Celtiberica bucks 1.16 ml, Jimenez-Rabadan *et al.,* (2013), Boer bucks 1.15 ml, Yodmingkwan *et al.,* (2016), Majorera bucks 1.21±0.03 ml, Batista *et al.,* (2009) and Blanca de Rasquera bucks 1 ml in 1 year and 1.8 ml in 2 year old Tabarez *et al.,* (2017). Seminal plasma contributes to about 70 per cent of the volume of the ejaculate. An increase or decrease, in the semen volume mainly depends on the quantities of fluids secreted by the epididymis and the accessory glands.

The colour of buck semen varies from pale yellow to cream colour (Bag *et al.,* 2002). Accordingly, the colour of semen in Chegu breed is abided by the latter findings.

Table 1: Average (Mean ±SEM) gross seminal parameters of Chegu bucks (n=6).

Parameters	Mean ±SEM
Volume (ml)	0.85±0.07
Colour	Creamy white to yellowish
Concentration (x10^6 spz/ml)	2238.5±231.0
Mass/Initial motility	3.92±0.03

The concentration of sperms observed in Chegu bucks (2238.5±231.0 x10^6 spermatozoa/ml; Table 1) was higher than 1759.3± 79.79 x10^6 spermatozoa/ml observed earlier in same breed by Thakur *et al.,* (2005b) and West African Dwarf bucks 1.77 x10^9 spermatozoa/ml, Waidi *et al.,* (2007); but lower than Blanca-Celtiberica bucks 2845±142.64 x10^6 spermatozoa/ml, Jimenez-Rabadan *et al.,* (2013), Gaddi bucks 3401.0± 247.2 x10^6 spermatozoa/ml, Sharma, (2018), Blanca Andaluza bucks, 3336-7776 x10^6 spermatozoa/ml, Gallego-Calvo *et al.,*(2015) investigated during different seasons.

The average mass motility was 3.92±0.03 in present study (Table 1). Previous reports are suggestive of lower (2.5 in 1 year old bucks, and 3.8 in >2 yr old Blanca de Rasquera bucks), Tabarez *et al., (*2017), 3.89±0.04, Gaddi bucks Sharma, (2018) and higher values of 4.33±0.08, in same breed, Thakur *et al.,* (2005b) for mass motility in bucks.

Average post thaw progressive motility observed in present study (Table 2) was higher than observations in Jamunapari bucks (28.82±1.99), Ramachandran *et al.,* (2015); Blanca de Rasquera bucks (18.2-24.5%); Tabarez *et al.,* (2017); Boer bucks (34.89±0.68), Yodmingkwan *et al.,* (2016); Gaddi bucks (35.18±0.87), Sharma, (2018); Jakhrana bucks (35.2±2.40), Priyadharshini *et al.,*(2011) and less than Jakhrana bucks (40.17-42.79%) Kumar *et al.,* (2016); Black Bengal bucks (40.89±0.65), Singh *et al.,* (2016); Alpine Sannen and Beetal crosses (45-56%), Narwade *et al.,*(2017); Chegu bucks (53.43±1.19), Thakur *et al.,* (2005b); Sannen bucks (47.50-55.63%), Niyazi *et al.,* (2014). Variation among present and other studies on post thaw motility could be due to subjective assessment of progressive motility, in present study only the progressively motile sperms with vigorous linear movement were included. Changes in the osmotic pressure during semen processing for cryopreservation critically affect the spermatozoa. This may be the most important deterrent to sperm survival during cryopreservation. It is most likely that the cryo damages are due to the irreversible destruction of individual components

Table 2: Average (Mean±SEM) fresh diluted and post thaw (n=100 ejaculates) seminal parameters of Chegu bucks.

Parameters(%)	Fresh diluted (Range)	Post thaw (Range)	Pvalue	% change due to processing
Progressive motility	75.48±0.69^B	37.38±0.90^A	0.000	50.4
Viability	75.79±0.95^B	48.25±1.78^A	0.000	36.3
Morphological abnormalities	5.64±0.29^A	7.02±0.32^B	0.002	19.6
HOST reactive	64.07±1.75^B	43.35±1.79^A	0.000	41.2

$^{A-B}$ Values with different superscripts within same row differs (P<0.01)

Table 3: Individual buck fertility to AI in Chegu does (n=40).

Buck No	Does inseminated (Number)	Does pregnant (Number)	Conception rate (%)	P value
2407	14	6	42.85	0.98
2408	16	7	43.75	
2417	10	4	40.0	
Overall	**40**	**17**	**42.5**	

of the structural organization of sperm cells Neild *et al.,* (2005).

Average post thaw viability observed in present study (Table 2) was higher than observations in Boer bucks (27.33±0.85), Yodmingkwan *et al.,* (2016); Jamunapari bucks (41.01±3.02), Ramachandran *et al.,* (2015); Gaddi bucks (45.26±1.32), Sharma, (2018) and less than Black Bengal bucks (50.48±0.65), Singh *et al.*,(2016); Jakhrana bucks (53.4±0.80), Priyadharshini *et al.,* (2011); Alpine Sannen and Beetal crosses (55.25-65.75%), Narwade *et al.*, (2017); Chegu bucks (65.20±1.53), Thakur *et al.*, (2005b). Similarly, HOST reactive sperms in present study (Table 2) were higher than observations in Boer bucks (8.04±1.02), Yodmingkwan *et al.,* (2016); Jamunapari bucks (41.01± 3.02), Ramachandran *et al.,* (2015); lower than Black Bengal bucks (47.02±0.58), Singh *et al.,* (2016), Jakhrana bucks (46.45-47.31%), Kumar *et al.* (2016); Jamunapari bucks (51.83±1.9), Ranjan *et al.*,(2015); Gaddi bucks (52.48±1.43), Sharma, (2018) and Alpine Sannen and Beetal crosses (61.0-64.63%), Narwade *et al.,* (2017), respectively.

The average morphological abnormalities in post thaw semen of present study (Table 2) corroborated with the findings of Elsheikh and Elhammali, (2015), 4.9-11% during different seasons in Sannen bucks; were lower than those of Sharma, (2018), 7.93±0.28%, Gaddi bucks; Kumar *et al.,* (2016) 12.18-13.73%, Jakhrana bucks; Singh *et al.,* (2016) 13.37-16.81%, Black Bengal bucks; Thakur *et al.*,(2005b) 23.3% Chegu bucks and much higher than those of Yodmingkwan *et al.,* (2016) 1.54-1.71%, Boer bucks; Ramachandran *et al.,* (2015) 2.84±0.49 Jamunapari buck, respectively. The most probable reason for morphological abnormalities seems to be the physical and chemical environments to which a spermatozoa is exposed during the preservation. On the other hand, Medeiros *et al.,* (2002) and Ozkavukcu *et al.,* (2008) observed that the sperm cell water exchange during the early stages of the preservation causes swellings and shrinkages which may be intolerable for the majority of organelles and might predispose spermatozoan to morphological abnormality.

In Chegu, overall nineteen kids were delivered (males: 7, 36.8%; females 12, 63.2%) with 1.11 kids per doe. Similar findings with 1.12 kids per doe in Gaddi goats Sharma, (2018) and higher were earlier observed in Saanen goats (1.78), Gacitua and Arav, (2005), Jamunapari goats 1.64, Kharche *et al.,* (2013) and 1.27 by Bhatacharya *et al.*,(2012). Twinning was evident in two does (11.8%) in present study. Indian studies, with the use of frozen thawed semen, have reported fertility rates varying from 35.8 to 53.3 per cent (Tiwari and Bhattacharya, 1988; Kharche *et al.,* 2013; Sharma, 2018) in different breeds of goats. In the study undertaken in exotic goats the fertility rates vary from 38 to 65 per cent (Gacitua and Arav, 2005; Dorado *et al.,* 2010). Variation in fertility between present (Table 3) and other studies could be due to differences in freezability, results of chilling injury Drobnis *et al.,* (1993) and fertilizing capacity of semen. Some males are more affected by cryoinjury than others which is probably attributable to their membrane biophysical and biochemical properties Arav *et al.,* (2000), season (Leboeuf *et al.,* 2000; Salvador *et al.,* 2005; Gangwar *et al.,* 2016), breeds (Leboeuf *et al.,* 1998; Salvador *et al.,* 2005; Nordstoga *et al.,* 2010; Sharma, 2018), reproductive status of female Dorado *et al.*,(2007) and site of semen deposition (Salvador *et al.,* 2005; Nordstoga *et al.,* 2010) could be some other variables affecting fertility rates. Studies in goats suggested increased pregnancy rate by increasing depth of semen deposition while using frozen–thawed semen Leboeuf *et al.,* (2000). Viudes-De-Castro *et al.,* (2009) used 200 IU of oxytocin simultaneous to AI to increase depth of semen deposition but neither kidding rate nor prolificacy was improved. The timing between semen deposition and ovulation is also an important factor in obtaining good conception results Maria and Dolors, (2014). In present study, artificial insemination was done in synchronized estrus with double straw along with intra-muscular injection of 4 µg Buserelin acetate. GnRH application during the estrus synchronizes the ovulation more precisely and thus improves the success of AI in fixed time insemination in does Pierson *et al.,* (2003). Stanimir *et al., (*2016) recorded pregnancy rate of (58.3%, single AI and 63.6%, double AI) after estrus synchronization in comparison to 37 per cent in natural estrus in Bulgarian White milk goats.

CONCLUSION

Semen cryopreservation along with artificial insemination can be effectively utilized to improve the population of endangered Chegu goats.

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