BJNRD (2017), 4(2): 1-10
Bhutan Journal of Natural Resources & Development

Article

www.bjnrd.org

Open Access

ISSN 2409–2797 (Print)
ISSN 2409–5273 (Online)

https://doi.org/10.17102/cnr.2017.05

Preliminary Studies of Seminal Attributes and Semen Cryo-banking of Nepali Indigenous Lulu (*Bos taurus*) Bull under Ex-situ Conservation

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Abstract

Indigenous animals are valuable genetic property of a country. The Nepali indigenous Lulu cattle breed is in endanger state. Cryopreservation is a simple and potent technology to preserve the gametes and could be used for genetic dissemination. Therefore, the study was conducted to evaluate the seminal attributes of fresh semen with a final goal of cryo-banking of Lulu (*Bos taurus*) semen. Two Lulu bulls were used for the study. A total of 24 ejaculates, 12 ejaculates from each Lulu bull were evaluated for seminal attributes. The mean semen volume, color, mass activity, sperm motility, sperm viability, sperm concentration, abnormal acrosome, mid piece and tail and, abnormal head were 1.6 ± 0.8 ml, 2.8 ± 0.7 grade, 3.1 ± 0.6 grade, 67.5 ± 9.6%, 90.7 ± 4.0%, 977.7 ± 76.1 x 10^6/ml, 10.1 ± 1.9 and 9.0 ± 1.4%, respectively. Further, 4 ejaculates (≥ 1.5 ml semen volume, ≥ 65% sperm motility and ≥ 800 × 10^6/ml sperm concentration) from each Lulu bull were used for freezing. The mean motility and viability were 71.9 ± 5.3% and 87.9 ± 6.7% after 90 minutes of cooling. Similarly, mean motility and viability were 70.0 ± 4.6% and 87.1 ± 6.3% after 210 minutes of cooling. The motility and viability of post thawed semen were 51.3 ± 5.2% and 70.4 ± 6.3%. A total number of 174 doses semen straw were cryo-banked. It was concluded that the semen volume, sperm motility and sperm concentration were comparatively lower compared with most of the bulls breed. The sperm viability and abnormal percentage was considered acceptable. Due to the acceptable post thawed sperm motility and viability recorded, cryopreservation of Lulu semen is hereby recommended so as to preserve the Lulu breed. Although, the cryopreserved semen must be tested in fertility trials following artificial insemination.

Keywords: Cryopreservation, Nepali indigenous Lulu bull, Semen

Introduction

Lulu is the only hump-less (*Bos taurus*) indigenous cattle breed of Nepal. They are found at high altitude (2800-4000 meters above sea level) of Trans-Himalayan districts; Mustang, Dolpa and Manang of Nepal (Neopane and Pokharel, 2005). The peculiarities of this breed are their small size having adult body weight 124.7 ± 1.8 kg, height at wither 87.7 ± 0.7 cm and body length 99.4 ± 1.2 cm - perhaps the smallest cattle breed in the world (Joshi *et al.*, 2015). The low milk producing capacity (1.6 ± 0.31 liter; Neopane e*t al.*, 2002) of Lulu, introduction of exotic breeds, indiscriminate cross breeding and lack of government attention results in genetic dilution. The number of this

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Received Oct, 2017 Accepted March, 2018

breed is decreasing gradually and has reached below 1000 animals throughout Nepal (Neopane and Pokharel, 2005; Joshi *et al.*, 2015). The breed is in endanger state. Indigenous animals are the valuable genetic property of a country. The unique characteristics of this breed need to be conserved and explored in future generations. One of the very simple technology to alleviate this problem temporary is cryopreservation of gametes. Semen cryopreservation is a simple and potent technology to disseminate genetic materials, accelerates breed improvement and productivity in bare minimum time. However, this technology needs preliminary study of seminal attributes, dilution, cooling and freezing of semen. Seminal attributes is the basic step towards semen cryopreservation to ensure maximum fertilizing capacity (Verstegen *et al.*, 2002; Dhurvey *et al.*, 2012). Success of semen cryopreservation depends upon several factors such as semen quality, composition of diluents, dilution-cooling-freezing and thawing protocol (Colas, 1975; Fisher and Fairfull, 1984; Pontbriand *et al.*, 1989; Medeiros *et al.*, 2002). In Nepal, no study have been conducted on Lulu semen till date. Therefore, the present study was preliminary designed to evaluate the seminal attributes, results of cooling, post thaw sperm quality and cryo-banking of indigenous Lulu semen.

Materials and Methods

This study was carried out over a period of 4 months (November, 2014 to February, 2015) at Animal Breeding Division (27.66º N and 85.35 E; altitude of 1400 metre above sea level), Nepal Agricultural Research Council, Kathmandu, Nepal.

Animal and management

Two sexually matured healthy Lulu bulls (Figure 1) managed under *ex-situ* conservation, aged 3.5 and 4 years, weighing 140 and 155 kg, body condition score 4 and 4.5 and scrotal circumference 28.4 and 30.6 cm, were selected. They were provided 8.0 kg green grass, 1.0 kg straw and 1.5 kg commercial concentrated feed per head per day with the provision of drinking water all the time. They were vaccinated against foot and mouth disease (FMD) and dewormed with Ivermectin twice yearly.

Reagent and stain preparation

The reagents and stains were prepared as described by Jha *et al.* (2013). Buffered formol saline was prepared by dissolving 6.2 gm disodium hydrogen phosphate, 2.5 gm potassium dihydrogen phosphate, 5.4 gm sodium chloride and 175 ml concentrated formaldehyde in 1,000 ml of double distilled water. William's stain was prepared as: Stock solution-I by dissolving 10 gm of basic fuchsin in 100 ml of 95% alcohol. Stock solution-II was prepared by dissolving a saturated solution of bluish eosin in 95% alcohol. Stock solution-III was prepared by mixing 10 ml of stock solution-I with 170 ml of 5% phenol solution. The final working solution contained 25 ml of stock solution-II and 50 ml of stock solution-III. Eosin-Nigrosin stain was prepared by dissolving10 gm of nigrosin, 1.7 gm eosin and 2.9 gm of sodium citrate in 100 ml of double distilled water. All these reagents and stains were filtered before use.

Extender preparation

Egg Yolk Tris Citrate extender was used for semen dilution (Ramukhithi *et al.*, 2011). Final extender was prepared into two fraction (Part A and Part B) using the following ingredients: 1.221 gm Tris, 0.68 gm citric acid monohydrate, 0.5 gm monohydrate glucose, 7 ml glycerol (only in extender Part B), 40 ml double distilled water (Part A) and 33 ml double distilled water (Part B). After proper mixing, extenders were autoclaved, brought to room temperature and stored at 5 °C. In the morning on the day of semen collection, 10 ml fresh hens yolk, 50,000 IU Penicillin G and 0.05 gm Streptomycin sulfate were added separately to each part; mixed thoroughly and maintained at 35 °C.

Semen collection and evaluation

Semen was collected and evaluated as described by Jha *et al.* (2013). Bulls were trained to mount homosexually to ejaculate semen into artificial vagina (AV). Semen was collected twice a week between 6.00 to 6.30 AM for a period of 2 months. Semen was collected following 2 successive false mount. The semen containing tube was maintained at 35 °C in a water bath for evaluation. The semen volume was recorded and color was scored visually into 4 grades. The mass activity was estimated by placing a drop (5 µl) of semen on a pre-warmed (35 °C) microscopic slide and scored into 4 grades without using cover slip under microscope (40 ×). The sperm concentration (10^6/ ml) was calculated by haemocytometer technique at the dilution rate of 1:100. Acrosome, mid-piece and tail was examined by wet mount technique using buffered formol saline. A drop (10 µl) of diluted formol saline fixed semen was placed on a microscopic slide and observed under microscope (1,000 ×). Sperm head morphology was examined by william's staining technique. A thin and dried semen smear was prepared and stained with carbol fuschsin for 8 minutes. The slide was rinsed gently in running tape water, dried off and examined under microscope (1000×). Sperm viability was estimated by using eosin-nigrosin stain. A thin and dried semen smear was prepared by mixing a large drop (10 µl) of eosin-nigrosin stain and a small drop (2 µl) of semen. The sperm that were alive exclude the stain; and dead stained with eosin against the dark nigrosin background (400 ×). At least 200 spermatozoa were examined from each smear. Photographs were captured by eyepiece camera (Coslab TM, Digital Camera, MDCE-5C, China) equipped with the microscope.

Semen dilution and cooling

Semen dilution and cooling was performed as described by Naing *et al.* (2011), with some modification. Only ejaculates having ≥ 1.5 ml semen volume, ≥ 65% sperm motility and ≥ 800 × 10^6/ml sperm concentration were considered for dilution and freezing. Semen dilution was done using two steps to obtain final sperm concentration of 25 × 10^6/ 0.25 ml French mini straw. Soon after evaluation, calculated volume of extender Part A was mixed with the semen. The diluted semen with extender Part A and calculated volume of extender Part B were transferred to cold handling cabinet (5 °C). After 90 minutes of cooling, calculated volume of extender Part B was mixed. The final diluted semen suspension was loaded into straws, sealed with poly vinyl chloride (PVC) powder and left further for 210 minutes for glycerol equilibration. The semen was evaluated for motility and viability at every 90 minutes and 210 minutes of cooling.

Freezing and thawing of spermatozoa

Freezing of semen was done by exposing in liquid nitrogen vapor in a Styrofoam box as described by Ramukhithi *et al.* (2011), with some modification. Following glycerol equilibration, the semen straws were arranged horizontally onto rack. The straw rack was held at 4.5 cm above the level of liquid nitrogen for 12 minutes within the Styrofoam box with its lid closed. The semen straw were plunged into liquid nitrogen, transferred into goblet and stored in Cryocan. Semen thawing was carried out in a water bath at 37 °C for 20 seconds. Two-three semen straws per batch were thawed to examine sperm motility and viability and data were averaged.

Statistical analysis

Descriptive analysis for the mean and standard deviation was done with the help of MS Excel in computer.

Results

Seminal attributes of fresh semen

A total of 24 ejaculates; 12 ejaculates from each Lulu bull were evaluated for volume, color, mass activity, motility, viability, concentration and abnormal sperm morphology. The results of seminal parameters are reflected in pooled mean and range (Table 1, Table 2). The

Table 1: Seminal attributes of fresh Lulu bull's semen (Mean±SD)

Bull ID	Volume (ml)	Color	Mass activity	Motility (%)	Viability (%)	Concentration (x10^6/ml)	Abnormal acrosome, mid-piece & tail (%)	Abnormal head (%)
L-111	1.6±0.6	2.7±0.8	2.8±0.5	63.8±10.7	87.6±2.9	945.0±121.1	10.3±2.1	9.36±1.6
L-112	1.6±1.0	2.8±0.6	3.5±0.5	71.3±6.8	93.8±1.9	1,010.4±218.7	9.9±1.7	8.8±1.3
Pooled	1.6±0.8	2.8±0.7	3.1±0.6	67.5±9.6	90.7±4.0	977.7±176.1	10.1±1.9	9.0±1.4

mean semen volume was 1.6 ± 0.8 ml and ranged from 0.5 to 3 ml. The semen color varied from water to milky white (scored into 1-3 grades). The mean sperm mass activity was scored as grade 3 and ranged from 2-4 grades. The mean sperm motility was 67.5 ± 9.6% and ranged from 45-80%. The mean sperm viability was 90.7 ± 4.0% and raged from 84-97%. The live and dead spermatozoa are shown in Figure 2. The mean sperm concentrations was 977.7 ± 176.1 x 10^6/ ml and ranged from 800.56 to 1368.36 × 10^6/ ml. The sperm in Neubauer counting chamber is shown in Figure 3. The mean sperm acrosome, mid-piece and tail abnormalities was 10.1 ± 1.9% and ranged from 7-17%. Whereas, the mean sperm head abnormalities was 9.0 ± 1.4% and ranged from 5 to 13%. The sperm abnormalities were recorded simple bent tail (Figure 4), coiled tail (Figure 5), decapitated head (Figure 6), pear shaped head (Figure 7), double mid-piece (Figure 8), giant head (Figure 9) and flat head (Figure 10).

Table 2: Seminal attributes of fresh Lulu bull's semen (minimum and maximum value)

Bull ID	Volume (ml)		Color		Mass activity		Motility (%)		Viability (%)		Concentration (x10^6/ml)		Abnormal acrosome, mid-piece		Abnormal head (%)	
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.
L-111	1	2.7	1	3	2	3	45	80	84	94	800.56	1,150.28	11	17	9	13
L-112	0.5	3	1	3	3	4	60	80	91	97	628.24	1,368.36	7	13	5	11
Pooled	0.5	3	1	3	2	4	45	80	84	97	800.56	1,368.36	7	17	5	11

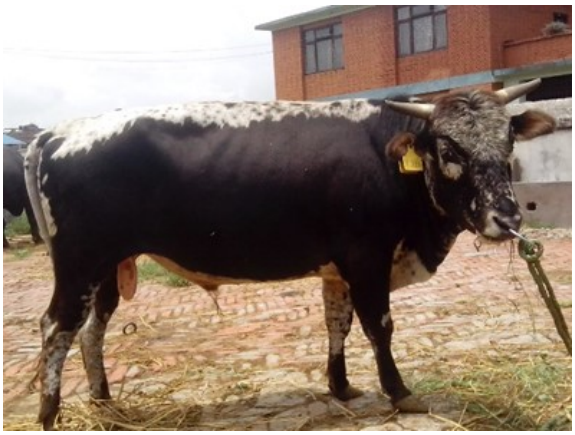

Figure 1: Lulu bull

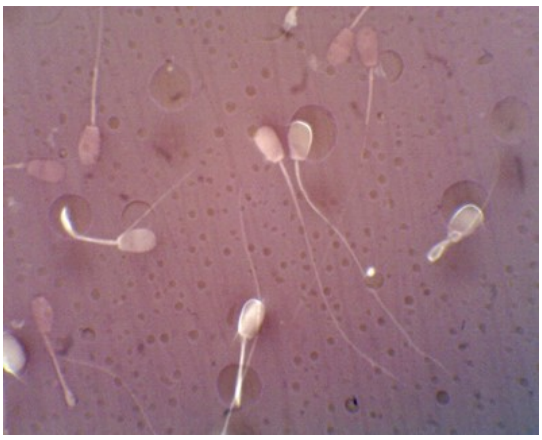

Figure 2: Live and Dead sperm (Eosin-Nigrosin staining, 400×)

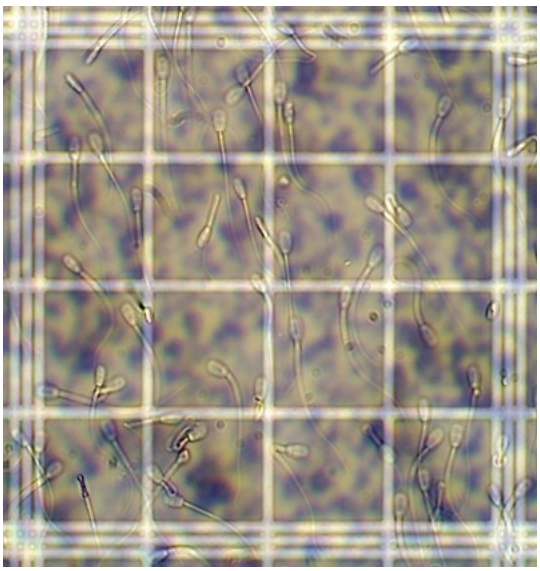

Figure 3: Sperm concentration 400×; Neubauer counting chamber)

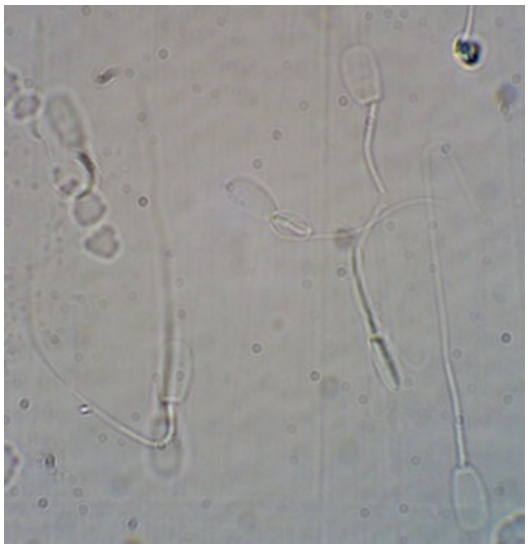

Figure 4: Simple bent tail (Buffered formol saline, 1000×)

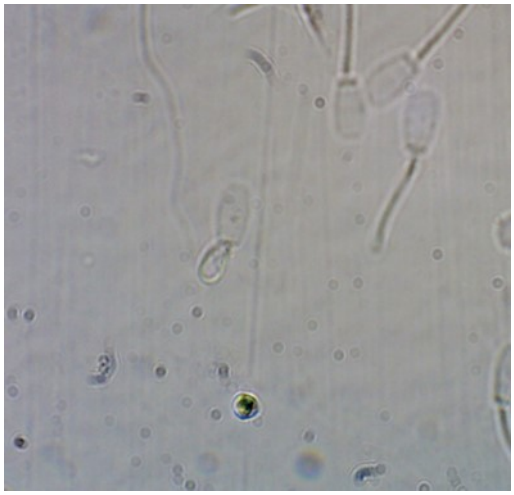

Figure 5: Coiled tail (Buffered formol saline, 1000×)

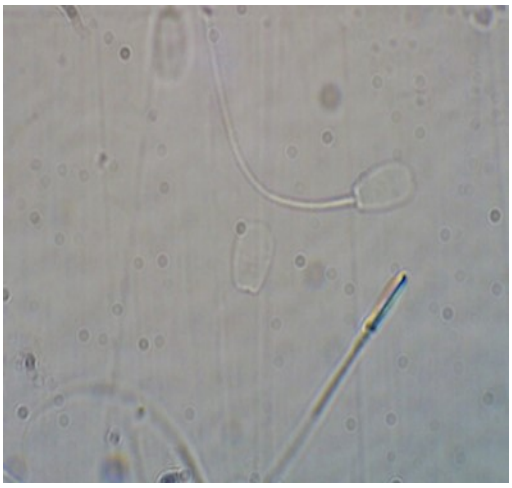

Figure 6: Decapitated head (Buffered formol saline, 1000×)

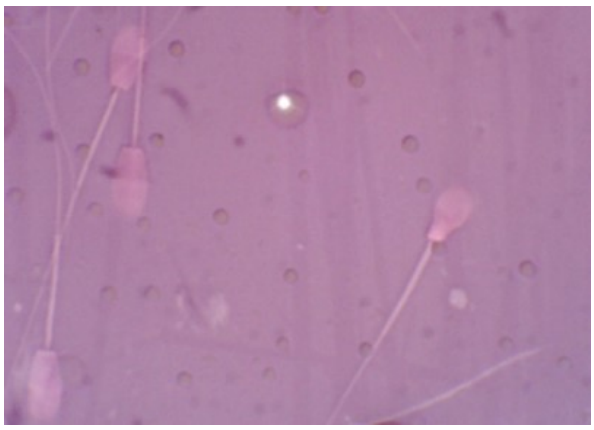

Figure 7: Pear shaped head (William's staining, 400×)

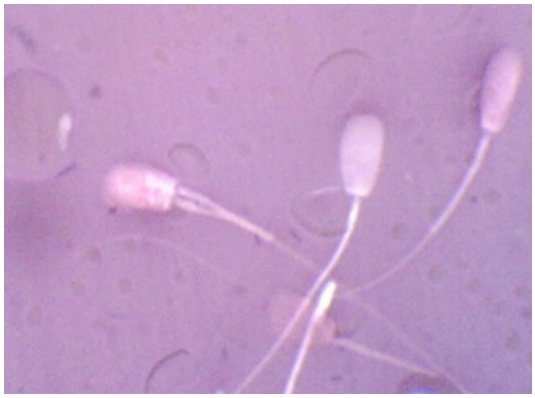

Figure 8: Double mid-piece (William's staining, 400×)

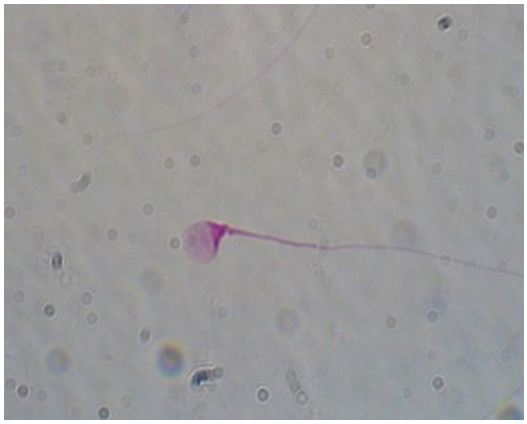

Figure 9: Giant head (William's staining, 1000×)

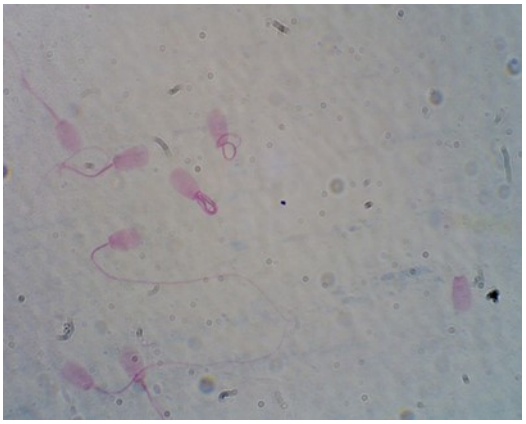

Figure 10: Flat head (William's staining, 1000×)

Table 3: Post- dilution motility and viability of Lulu bull's sperm cells at 5 °C (Mean±SD)

Bull ID	90 minute of cooling (Semen + Extender Part A)		210 minute of cooling (Semen + Extender Part A + Extender Part B)	
	Motility (%)	Viability (%)	Motility (%)	Viability (%)
L-111	67.5±2.9	87.3±3.0	66.3±2.5	86.8±2.8
L-112	76.3±2.5	88.5±9.7	73.8±2.5	87.5±9.1
Pooled	**71.9±5.3**	**87.9±6.7**	**70.0±4.6**	**87.1±6.3**

Semen dilution and cooling

A total of 8 ejaculates; 4 ejaculates from each Lulu bull were selected for semen dilution and cooling. The results of post-dilution sperm motility and viability of sperm cells are reflected in pooled mean (Table 3). The mean sperm motility was 71.9 ± 5.3% and 70.0 ± 4.6%, and the mean sperm viability was 87.9 ± 6.7% and 87.1 ± 6.3% each at 90 and 210 minutes of semen cooling. The sperm motility and viability decreased with advancement of cooling time.

Post thawed semen quality

The result of post-thawed sperm motility and viability were reflected in pooled mean and are shown in Table 4. The mean sperm motility and viability were 51.3 ± 5.2% and 70.4 ± 6.3%, respectively.

Cryo-banking

A total of 174 doses semen straw were cryo-banked at Animal Breeding Division, Nepal Agricultural Research Council, Nepal.

Table 4: Post- thawed motility and Viability of Lulu bull's sperm cells (Mean±SD)

Bull ID	Motility (%)	Viability (%)
L-111	48.8±2.5	66.3±2.1
L-112	53.8±6.3	74.5±6.6
Pooled	**51.3±5.2**	**70.4±6.3**

Discussion

Seminal attributes of fresh Lulu bull's semen

Seminal attributes studied included volume, color, mass activity, sperm motility, sperm viability, sperm concentrations and abnormal sperm morphology The semen volume was lower than those reported 4.32 ± 0.24 ml in Cross bred (Chauhan *et al.*, 1983), 3.40 ± 1.30 ml for indigenous non-descript zebu (Siddiqui

et al., 2008), 3.10 ± 0.35 ml in semi wild Mithun (Bhattacharyya *et al.*, 2009), 3.25 ± 0.15 ml in Punganur bulls (Bramhaiah, 2012) and 9.25 ± 2.5 ml in Gir bulls (De Lucio *et al.,* 2014), respectively. The normal color of bull semen is milky white or light yellow. The mass activity recorded in our study is consistent with Fiaz *et al.* (2010) who reported mass activity of 2.21 ± 0.04 to 3.15 ± 0.12 (scale: 0-5) in Jersey bull. Similarly, our finding of mass activity is comparable with Singh and Pangawkar (1990) who reported 3.10 ± 0.54, 3.00 ± 0.11 2.88 ± 0.08, 2.83 ± 0.06 and 2.80 ± 0.06 (scale: 0-4) in different Holstein Friesian (HF) and Jersey cross bulls. The sperm motility found in our study is comparable with Chauhan *et al.* (1983), Farooq *et al.* (2013) and De Lucio *et al.* (2014) who reported 64.2 ± 0.88% to 59.86 ± 0.76% in crossbred bulls, 64.38 ± 2.64% in Cholistani bulls, 62.10 ± 5.8% in Gir bulls and 65.20 ± 3.9% in Nellore bulls. In contrast, Bramhaiah (2012) who reported 72.85 ± 0.71% sperm motility in pure breeds. The sperm viability in our study is within the range 80.6 ± 10.7 to 92.4 ± 6.3% in pure Jersey bulls (Sugulle *et al.*, 2010). The sperm concentrations in our present study are comparable with Farooq *et al.* (2013) who reported 1062.22 ± 101.26×10^6 /ml in Cholistani, 890.47 ± 18.45 to 995.16 ± 7 0.99×10^6/ ml in Jersey. In contrast, De Lucio *et al.* (2014) recorded higher sperm concentration of 1473.90 ± 343.2 and 1662 ± 343.80×10^6/ ml in Gir and Nellore bulls. The total abnormal sperm count in our study is within the range reported by Vilakazi and Web (2004), Farooq *et al.* (2013) and De Lucio *et al.* (2014) who reported 20.30 ± 1.3% in Friesland bulls, 16.03% in Cholistani bulls and 17.0 ± 9.0% in Gir bulls. The average quality of semen should not contain more than 20% abnormal sperm, while semen containing more than 30% abnormal sperm would be considered as poor (Hafez, 1993). The differences in seminal parameters might be due to breed, age, body weight and nutritional status (Almquist *et al.,* 1979). Variations also attributed by season of collection, sexual excitement, method and frequency of semen collection (Lagerlof, 1934). The color of semen mainly vary due to presence of pigment lipochrome or riboflavin (Hafez, 1980; Patel and Siddiquee, 2013). The difference in sperm concentration is due to individual genetic variation. The percentage of live or dead sperm may be attributed due to frequency of collection, age of breeding bull and season. The quality of bull semen might be affected by genetic or non-genetic factors like temperature, photoperiod, humidity and nutrition (Haugan *et al.*, 2005).

Semen dilution and cooling

The post dilution sperm motility in our study is in agreement with Talluri *et al.* (2011) and, Patel and Siddiquee (2013) who reported 69.55 ± 1.47% in Ongole and 74.73 ± 0.58% in Kankrej bulls. In contrary, Salvador *et al.* (2008) found higher post dilution sperm motility who reported as 66.45 ± 5.7% in Nellore bulls. The variation may occur due to different type of extender including the concentration of glycerol and egg yolk used for processing semen (Gaillard and Kupferschmied, 1982). The reduction of live and motile sperm percentage could be attributed to the death of the spermatozoa due to cold shock which is attributed as a result in rate of cooling and glycerol equilibration time during processing of semen for freezing semen (Bailey *et al.*, 2000). Cold

Post thawed sperm quality

The post thawed sperm motility in this study is in agreement with Mishra *et al.* (2012) and Kedia *et al.* (2014) who reported 49.64 ± 2.06% to 51.16 ± 1.08% in Jersey and Sahiwal bulls. In contrast, Rana and Dhami (2004) reported 46.00 ± 1.16% in Gir bulls. Higher post thaw motility was reported 54.02 ± 2.71 to 57.85 ± 1.61 in different pure bull breeds (Farooq *et al.*, 2013; Patel and Siddiquee, 2013). The post thaw sperm viability is in agreement with Nath *et al.* (1996) who reported 66.50 ± 2.25% in pure Jersey bulls. In contrast, Farooq *et al.* (2013) reported much higher post thawed viability 71.89 ± 2.05% in Cholistani bulls. Whereas, Veeraiah *et al.* (1999)

and Salvador *et al.* (2008) reported much lower post thawed viability 44.69 ± 1.00% and 34.50 ± 11.1% in Nellore and Ongole bulls. The result of post thaw semen quality depends upon several factors like type of extender used for processing semen, the amount of glycerol included in the extender, the rate of cooling used, equilibration period, packaging and rate of freezing and thawing of the semen (Gaillard and Kupferschmied, 1982). The motility and viability of sperm decreased to some extent during cooling is might be due to cold shock, it can be observed as a permanent loss of motility in the thawed sperm. Cold shock usually takes place when the semen temperature rapidly decreases from approximately body temperature of the bull (35 °C), to the freezing temperature of water (0 °C) (Bailey *et al.*, 2000; Medeiros *et al.*, 2002). The quality of semen deteriorates as the spermatozoa undergoes several ultrastructural, biochemical and functional changes during freezing and thawing (Salamon and Maxwell, 2000).

Conclusion

The semen volume, sperm motility, and sperm concentration were comparatively lower when compared with most of the bull breeds. Due to the acceptable post thawed sperm motility and viability recorded, cryopreservation of Lulu semen is hereby recommended so as to conserve the Lulu breed. However, the cryopreserved semen must be tested in fertility trials following artificial insemination.

Acknowledgements

The authors wish to acknowledge the Nepal Agricultural Research Council (NARC) for financial, laboratory facilities and providing the Lulu bulls.

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