Preservation and evaluation of Jamunapari buck semen and it's fertility after artificial insemination in goats

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Roll No. : 0119/01

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Authorization

The work presented in this thesis is entirely my own and I hereby declare that I am the sole author of the thesis entitled "**Preservation and evaluation of Jamunapari buck semen and it's fertility after artificial insemination in goats**". I also declare that it has not been previously submitted to any university for the award of a degree.

I, the undersigned, and author of this work, declare that the electronic copy of this thesis provided to the CVASU Library, is an accurate copy of the print thesis submitted, within the limits of the technology available.

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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DEDICATED TO MY PARENTS AND MENTORS

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The Author

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FAO	Food And Agriculture Organization
º C	Degree celsius
GDP	Gross Domestic Product
%	Percentage
AI	Artificial insemination
CAI	Cervical artificial insemination
µm	Micrometer
ANOVA	Analyis of variance
cm	Centemeter
g	Gram
hrs	Hours
mg	Milligrams
kg	Kilograms
ml	Milliliter
mm	Milimetre
µl	Microliter
TCEY	Tris citric egg yolk
JP	Jamunapari
EY	Egg yolk
DNA	Deoxyribonucleic acid
HOST	Hypo-osmotic swelling test
SDc	Skimmed milk D-glucose chill extender
SDw	Skimmed milk D-glucose buffer with deionized water
SDg	Skimmed milk D-glucose extender with glycerol
TCEYw	Tris citric egg yolk buffer with deionized water
TCEYc	Tris citric egg yolk chilled extender
TCEYg	Tris citric egg yolk with glycerol
SDf	Skimmed milk D-glucose frozen extender
TCEYf	Tris citric egg yolk frozen extender
Liq. N2	Liquid nitrogen

Abstract

Preservation of buck semen is necessary for rapid improvement in goat production. The goals of this study were to evaluate the quality of fresh semen from Jamunapari bucks, the effects of different semen extenders on the quality of preserved semen, and the fertility of goats using preserved semen after AI. Semen was collected from two adult Jamunapari bucks once a week using the Artificial Vagina (AV) method. Fresh semen from the two JP bucks was evaluated immediately after collection for quality assessment, such as volume, color, density, concentration, mass motility, pH, morphology of sperm, and functional integrity. Fresh semen from two bucks was pooled in a sterile falcon tube and processed for chilling and cryopreservation with Tris-citrate egg yolk (2.5% and 5% EY) and skimmed milk-based (SD) extenders. The effects of these extenders on preserved semen quality and fertility after AI in goats were observed. Fresh semen quality, volume (1.33±0.10 vs 1.06±0.15 ml), concentration of spermatozoa (3166.34±22.31 vs 2908.42±41.03×10^6/ml), sperm viability (85.06±0.21 vs 82.73±.73 %), normal sperm (94.73±0.20 vs 93.91±0.29 %) and HOST +ve sperm (82.53±0.18 vs 80.10±0.47 %) found different in between bucks ($p<0.05$). In chill semen preservation, 5% TCEYc extenders maintained better semen quality than 2.5% TCEYc and SDc extenders from day 1 to day 4 of storage ($p<0.05$). Moreover, semen quality decreased dramatically during storage at 4 °C ($p<0.001$). Similar to chilled semen, in frozen semen, 5 % TCEYf also maintained higher motility, viability, functional integrity, and normal morphology of spermatozoa compared with 2.5 % TCEYf and SDf, respectively, with different cryopreservation times: days 1, 5, 10 and 20th day of observation ($p<0.001$ for all). However, the preservation time did not affect semen quality for each extender during cryopreservation ($p > 0.05$). Cervical AI was performed in natural estrous goats using chilled semen, and no significant effect of extenders on PR was found ($p > 0.05$), with an overall PR of 49.12%. However, PR was significantly higher when CAI was performed with frozen semen extended with 5% TCEYf (46.42%) than with 2.5% TCEYf (32.14%) and SDf (26.92%) ($p<0.05$). The overall PR in goats using JP buck frozen semen extended with these three extenders was 35.36 %.

Key words: Jamunapari buck, semen, preservation, extender, evaluation, fertility

Chapter-1

Introduction

Goats are major livestock species that play an important role in the agro-based economy of Bangladesh. According to the BBS (2022), approximately 26.7 million goats are reared in Bangladesh, mainly by middle class people. Goats have massive socio-economic importance in developing countries, such as Bangladesh, as they are a potent food source and provide income to small households (Sahlu and Goetsch, 2005). Many goat breeds have been reared in Bangladesh. Among different goat breeds, Jamunapari, Black Bengal, and crossbred goats are available in this area (Bhowmik et al., 2014). In the livestock sector, Jamunapari goat is one of the recognized breeds available in Bangladesh. The Chattogram region of Bangladesh is home to a large number of goats. The hot and humid climate of the Chattogram makes it ideal for goat farming. The Jamunapari goat, called the duel-purposed breed, is the tallest breed and is famous for its high fertility, prolificacy, superior chevon quality, best quality skin, resistance against common diseases, seasonality, low kidding interval, milk production, and very good adaptability.

To improve the proliferation of goat species, an effective breeding strategy and breeding bucks are required. Unfortunately, there is a significant lack of breeding bucks in the country. The use of the same buck year after generation has increased the risk of inbreeding, resulting in reduced reproductive performance, as well as the spread of different venereal and infectious illnesses (Hussain, 2007). To overcome this situation, the development of effective breeding policies is needed (Jabbar et al., 2010). This can be accomplished by selecting and mating between superior males and females (Bhatia and Arora, 2005). Natural breeding is a limiting issue because a buck can mate with only a limited number of females, producing limited kids, whereas artificial insemination (AI) techniques that same buck can produce more offspring per year. To improve the genetic makeup of goats, it is important to study their reproductive efficiency, as this will enhance the proper selection of proven bucks. Among the reproductive traits, semen quality plays a major role in determining fertility and reproductive efficiency of livestock production (Moussa, 1997). The volume of ejaculate, sperm concentration, sperm motility, and proportion of living and normal spermatozoa influence the quality of semen in relation to fertility.

Artificial insemination (AI) has remained the primary vehicle for the rapid dissemination of valuable genes and has long been the method of choice for farmers worldwide seeking to increase the genetic quality of their livestock (Vishwanath, 2003). AI popularity in goats has not grown as much as in cattle because of the performance of the presrved buck semen (Herman et al., 1994). Therefore,the success of an artificial insemination (AI) program depends on proper management of semen collection, preservation, and use. Tris-egg yolk-fructose and non-fat dried skim milk extenders are most commonly used for the preservation of buck semen (Purdy, 2006). The egg yolk used in semen extenders plays a major role during the freezing steps of buck sperm cryopreservation (Aboagla and Terada, 2004) and provides sperm cells with nutrients, such as proteins (Ritar et al., 1990). However, egg yolk may have a negative effect on buck semen preservation because of the presence of phospholipase, an enzyme present in the seminal plasma secreted by the bulbourethral gland that catalyzes the hydrolysis of lecithin in egg yolk to fatty acids and lysolecithins, which are toxic to spermatozoa and cause coagulation of the storage medium (Iritani and Nishikawa, 1972). Phospholipase A, a specific enzyme found in goat sperm, interacts with milk present in extenders to produce chemicals that degrade sperm quality (Pellicer-Rubio and Combarnous, 1998). These enzymes are thought to interact more extensively with milk during cryopreservation and hydrolyze the triglycerides of the sperm plasma membrane. Skimmed milk produces oleic acid, a fatty acid that is toxic to spermatozoa (Pellicer-Rubio et al. 1997), resulting in a significant decrease in sperm velocity. This impact could possibly be linked to the high calcium content in milk, which may be responsible for increasing the activity of phospholipase A, which requires calcium to function and hence increases the membrane's permeability to ions (Martins, 2006). However, a protein discovered as SBUIII in the same gland was also found to decrease the survival of cooled or frozen goat sperm diluted in milk-based extenders (Nunes et al., 1982). The components that cause harmful effects were later identified as tricylglycerol lipase and 55–60 kDa glycoprotein lipase (BUSgp60) (Pellicer-Rubio et al., 1997; Pellicer, 1995). This ingredient resulted in a decrease in sperm motility and quality of movement in the skim milk extender. Cryopreserved sperm cells can be preserved for a long time before use. The cryopreservation of buck semen extends the reproductive life of a buck after his own life (Rahman et al., 2008). The commercial use of frozen-thawed semen in caprine reproduction is limited (Batisa et al., 2009), and further research is needed before it can be used more widely.

Freezing of caprine semen is technically challenging because it contains bulbourethral secretions capable of interacting with the cryoprotectants used, and the freezing and thawing processes result in reduced viability, motility, and velocity of sperm movement. This, in turn, decreases the conception rate, which varies from 30 to 70% when frozen-thawed semen is used for AI (Dorado et al., 2007). The anatomical arrangement of the cervix in goats and the difficulty of traversing it require a higher number of sperm cells to obtain a reasonable pregnancy rate via cervical insemination. Thus, a reliable means of insemination of goats with frozen semen would be most advantageous not only for commercial breeders interested in the genetic improvement of their stock, but also for small producers who cannot afford their own breeding stocks (Sohrney and Holtz, 2005). Information on different semen extenders for preservation and fertility of goats using jamunapari buck-preserved semen in AI is scarce.

Therefore, the present study was undertaken to observe fertility after AI in goats using preserved Jamunapari buck semen with the following objectives.

i. Evaluation of fresh semen in Jamunapari bucks.

ii. Effects of different semen extenders on the quality of preserved semen.

iii. Evaluation of fertility after AI in goats using preserved Jamunapari buck semen.

Chapter-2

Review of literature

Goats are common household animals in Bangladesh because of their ability to survive and produce despite adverse climatic and management situations. Goats are more effective than other animals in converting feed into meat and milk. Jamunapari goat is a large, dual-purpose breed that is popular in this area. Effective goat production is critical for reducing protein shortages and enhancing income creation. AI is the most effective and cost-efficient option for improving jamunapari goat production, with high genetic merit. Semen storage processes and semen extenders with appropriate components are essential for the success of AI in goats.

2.1. Jamunapari goat

Goats are multipurpose animals that play an important role in the rural economy of Bangladesh. It is vital to the survival of a large number of small farmers, notably women, landless farmers, and marginal farmers who live in geographically remote places and have few other options (Choudhury et al., 2012). There are many goat breeds in Bangladesh, including Jamunapari. Jamunapari goats are multipurpose animals that produce meat, milk, skin, and hair (Amin et al., 2001). It originated from the Chakarnagar Region of Uttar Pradesh (U.P), India, and is known as the Chambal Queen or Chakarnagar Pari. The coat color of jamunapari goats is mainly white, but white mixed light brown color is also found in different regions of Bangladesh. Body weight of adult jamunapari buck is 50.70 kg and does are 45.47 kg (Bhowmik et al., 2014). Age at sexual maturity in Jamunapari buck 9-12 months of age whereas female is 11-13 months of age (Hassan et al., 2010). Testis length, testis breadth and Scrotal circumference of JP buck are recorded as 17.3 cm, 11.5 cm and 42.4 cm, respectively (Hassan et al., 2010). The contribution of the livestock sector to the overall GDP was 1.66% for 2015–16, where the share of livestock in the total agricultural GDP was 14.21 (DLS, 2016). Like other domestic animals, goats play a significant role in the Bangladeshi economy (Faruque et al., 2016). Male goats that are used for breeding purposes are called bucks, and castrated males are called withers or khasi. The reproductive lifespan of a buck is longer than does. In Bangladesh, scientific breeding programs are absent; people use traditional inbreeding programs in urban and rural

areas, which reduce the genetic merits of goats. Very little information has been found regarding reproductive characteristics, semen preservation, and AI of Jamunapari bucks to enhance their genetic potential.

2.1.1. Management of Jamunapari goat

There are several factors that influence livestock reproductive fitness, including genetic merit, the environment (Kafi et al., 2004; Karagiannidis et al., 2000), husbandry and most importantly, the nutritional status of the animals (Al-Ghalban et al., 2004).

2.2. Goat spermatozoa

It is commonly known that most mammalian spermatozoa have a flattened head with an oval shape (Garner, 2006). The size and shape of spermatozoa varies depending on the species. Bull and human spermatozoa have paddle-shaped heads, rats have hooked-shaped heads, and chicken sperm have spindle-shaped heads that are nearly impossible to tell apart from the midpiece (Konobil and Neil, 1998). The isolated goat spermatozoa have slightly different dimensions from others. Head and acrosome are smaller in goats than others but midpiece and tail length is higher than other farm animals. The mean head length of goat spermatozoa is 10.04 µm; the width – 5.22 µm; the circumference – 27.18 µm, and the area – 38.69 µm2. The acrosome area is 23.07 µm2. Mid-piece and tail length was 17.03 µm and 77.97 µm respectively. The mean overall length of goat spermatozoa amounted to 88.0 µm. (Andraszek et al., 2014).

2.3. Evaluation of semen

Evaluation or analysis of semen is the most initial and essential parameters offering high accuracy and reliability of semen to produce AI dose. Spermatogenesis and fertility test in male is an essential part of andrology, which can only be confirmed through semen evaluation (WHO, 2010). It also helps to investigate the male disorders (WHO, 1999). Recently, there are several methods or techniques involved in the evaluation of semen. Many laboratories have adopted digitized semen analysis. Computer Aided Sperm Analysis (CASA) systems are most common (Gordon, 2010). However, such digital systems did not get more popularity because of technical

limitations and were expensive especially for the laboratories under developing countries (Pacey, 2010). Alternatively, different types of microscopic are the aid, which helps to investigate the spermatozoa. Light and phase contrast microscope is a common assist, which is used to investigate the spermatozoa for their evaluation. The parameters that include routine semen evaluation are volume, density, mass motility concentration, motility, viability, functional integrity test and morphology (Rowe et al., 2000; Novak et al., 2010). It is true that the ability to guess the fertility of semen with laboratory tests is still limited because of their complex structure (Januskauskas and Zilinskas, 2002). Recently more attention has been given to evaluate semen with applying more sophisticated techniques e.g., sperm evaluation with multicolor flow cytometry (Graham. 2001). Sperm physical characteristics including sperm size, shape and internal complexity, understanding the biochemical and functional status are also possible by various kinds of fluorochromes and compounds conjugated to fluorescent probes (Gillan et al., 2005). These techniques can also provide the information on sperm viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity, apoptotic mechanism and DNA status (Garner et al., 1997; Nagy et al., 2003). Although having many advantages of this technique, it is somehow quite impossible to apply everywhere because of expensive and operation limits. To compensate these hazard, sperm concentration, morphology, motility and viability (Rowe et al., 1993; Gundogan et al., 2011), sperm functional integrity test by hypo osmotic swelling (HOS) test method (Fukui et al., 2004), acrosome abnormality study (Baran et al., 2004), different concentration of egg yolk and glycerol on semen characters (Ranjan et al., 2015), extenders on semen characters (Gundogan et al., 2011; Kulaksiz et al., 2013) are the common and convenient study to semen evaluation (Salamon and Maxwell, 2000; Leboeuf et al., 2000).

2.3.1. Volume of semen

Semen volume is one of the important factors in semen evaluation and semen preservation in males (Ax et al., 2000). The volume of semen could vary with the breed, age, season of the semen collection, level of nutrition supplied to the buck, testicular size and the collection interval. The normal volume of buck semen is 0.5-1.5 ml (Hahn et al., 2019); and in rams, cattle, and buffalo, it is 0.3-1.0 ml, 2-10 ml, and 0.5-4.5 ml, respectively (Knobil and Neill, 1998; Moss et al., 1988). Sultana et al.

(2013) noted that the overall mean value of the semen of Black Bengal buck is 0.58±0.17 to 1.04±0.11ml. Whereas Apu et al. (2008) found the mean volume of Black Bengal buck semen was 0.58±0.03 ml. The Sirohi breeding buck produces 1.06 ± 0.01 mL of semen in the breeding season (Khadse et al., 2019). Hassan et al. (2010) found the average volume of Jamunapari semen was 0.9±0.2 ml. There is more variation in the volume of buck semen, but limited research was done to evaluate the volume of Jamunapri buck found in Bangladesh.

2.3.2. Concentration of spermatozoa

Concentration of spermatozoa in semen depends on age, breed, frequency of collection, number of ejaculation, season of the year. Faruque et al. (2007) who obtained average sperm concentration of 2828 ± 11.8×10^6/ml in Black Bengal buck. However, Goswami et al. (2020) found 3356.73± 83.06 million/ml in Beetal bucks and 3176.94±53.92 million/ml in Sirohi bucks which varied according to breed variation.

2.3.3. Motility of spermatozoa

Sperm motility is a fairly reliable indication of the viability of semen. It is most common parameter of semen evaluations, which indicate quality and viability of spermatozoa (Fonseca et al., 2005).The motile spermatozoa is the strong evidence of sperm matuaration. Rapid progressive motility has a significant impact on fertility because those spermatozoa can easily cross the cervix through cervical mucus (Bjorndahl, 2010). Yodmingkwan et al. (2016) found sperm motility of Boer buck fresh semen is 80.83 ± 3.06% and letter it reduced in frozen semen while extended with different extenders. Sultana et al. (2013) also found that following freezing, the fresh semen motility of Black Bengal buck was reduced by 77.07±1.06 to 81.47±1.84% percent (48.15±1.99 to 55.88±2.97%).

2.3.4. Viability of spermatozoa

Determination of percent (%) viable sperm is important in assessing semen for AI. The sperm membrane is damaged during preservation, resulting in membrane permeability loss and cell death (Croass and Hanks, 1991). Fresh sperm should have >70% viable spermatozoa in order to have a successful conception rate (Nilani et al., 2012). Various factors depend such as age, breed, improved diet, semen volume extender etc on sperm viability of fresh semen as well as in preserved semen of domesticated animals (Yodmingkwan et al., 2016 and Kadirvel et al., 2009). A preliminary study was performed by Hassan et al. (2010) in Jamunapri goat in Bangladesh and they recorded the percent sperm viability was 90.3 ± 2.2%. The mean proportion of dead spermatozoa in Black Bengal buck fresh semen was 14.4±038% to 15.01±0.52% (Hossain, 2007). After preservation with different semen volume extender the viability of spermatozoa of goat varied significantly (Mara et al., 2007).

2.3.5. Functional integrity of spermatozoa

Integration of HOST into the sperm selection method may provide a beneficial tool for selection of functional sperm. Recently, the hypo-osmotic swelling test has been proposed to potentiality select sperm with intact membrane. The hypo-osmotic swelling test was developed to evaluate the functional integrity of the sperm membrane (Jeyendran et al., 1984). Live spermatozoa with normal membrane functions show swelling and coiling of the tail due to water influx when exposed to hypo-osmotic conditions (Wallach and Baker, 1992; Avery et al., 1990). Rizal et al. (2018) reported that the overall mean hypo-osmotic swelling test value was 88.20±0.84% in Boer buck fresh semen and also observed a decrease in the hypo-osmotic resistance of spermatozoa with increasing storage period. Vera-Munoz et al. (2011) found 68.1% and 48.8% HOST positive sperm in fresh and post thawed semen in bull which affects the fertility. So, integration of HOST into the sperm selection procedure may provide a valuable tool for selection of functional sperm required for fertilization.

2.3.6. Morphology of spermatozoa

Morphology of spermatozoa means shape and structure. Morphological normal spermatozoa are important to determining fertility. There are different types of abnormality of spermatozoa recorded: macrocephalic, microcephalic, pear shape head, narrow head, stunted tail, bent tail, dag defect (Koonjaenak et al., 2007). In normal and mature spermatozoa should have neck and midpiece short and no cytoplasmic droplets while immature spermatozoa consist of cytoplasmic droplets attached to the midpiece area or tail (Knobil and Neill, 1998). However different factors affect the sperm morphology. Types of extenders, semen freezing increased the rate of sperm morphological abnormalities (Kulaksiz et al., 2013). For good quality semen the normal spermatozoa should be more than 70% in Ram and < 20% abnormal spermatozoa in bull semen (Saragusty et al., 2009; IAEA Manual, 2005). Apu et al, (2008) who reported 91.27±0.47 to 92.08±0.39% of normal sperm and the difference in buck to buck is non-significant. Sultana et al, (2013) found in Black Bengal buck 87.17±2.40 to 91.85±1.38 % normal spermatozoa in fresh semen. In Bangladesh, the Jamunapari goat produced semen with 94.3±3.5% normal sperm (Hassan et al., 2010).

2.4. Overview of semen preservation

Sperm cryopreservation together with different assisted reproduction techniques (ART's) such as artificial insemination allows us to use the semen from valuable sires, the preservation of endangered species, to solve problems of male infertility and exchange of semen between subpopulations that may become geographically or biologically isolated (Watson and Holt, 2001; Andrabi and Maxwell, 2007). The cryopreservation of mammalian sperm is a complex process that involves balancing many factors in order to obtain satisfactory results.The success of artificial insemination (AI) is based on the ability to efficiently collect and cryopreserve spermatozoa from quality bucks for use on does over generations (Amoah and Gelaye, 1990). Three methods of semen preservation (fresh, refrigerated and frozen) are used worldwide in goats (Leboeufet al., 2000).Smith and Polge, (1950) did pioneering work in goat semen cryopreservation. Barker (1957) was the first to propose that the fertility of frozen-thawed goat sperm was too low to be useful.

Different extenders and freeze techniques have been described in various animal species since then, particularly in bulls (Martin et al., 2004), goat (Jimenez-Rabadan et al., 2013 and Sharma et al., 2020) and ram (Munyai, 2012) to minimize detrimental effects of cryopreservation on spermatozoal motility, viability, normal morphology (Gravance et al., 1997). As spermatozoa are likely to suffer significant damage and deterioration during dilution and storage at low temperatures, proper diluents are a must for successful spermatozoa preservation and a greater conception rate in field trials using diluted semen (Salamon and Maxwell, 1995). Semen cryopreservation involves common steps to any protocol such as collection and extension of semen, addition of cryoprotectant, cooling above 0˚C, cooling below 0˚C, storage and thawing (Curry, 2007).

2.4.1. Semen collection and processing

There are different kinds of semen collection techniques such as artificial vagina (AV), electro-ejaculator (EE) and vaginal collection vial (VCV) used in different species such as bulls, rams, bucks and boars (Mahoete, 2010). For semen collection with the VCV, a glass vial of approximately 9 cm long, bending at 10° angle is inserted into the vagina of the doe about 10 min before mating (Mahoete, 2010). During mating, the buck will ejaculate in the VCV. However, the most preferred semen collection techniques in bucks are AV.

Artificial vagina (AV) is a technique which was first introduced by the Russian scientist. The AV briefly consists of a firm cylinder of elastic and a thin walled elastic tube for the inner lining. A water-tight jacket is formed inside the cylinder by folding both ends of the thin walled elastic tube over the outer cylinder. The water jacket is filled with warm water, (37 °C to 43 °C) to keep the inside temperature of the AV warm to a few degrees celsius above normal body temperature (Matshaba, 2010). The warm temperature from the water on the AV provides thermal and mechanical simulation over the glans penis for the buck to ejaculate (Matshaba, 2010 and Munyai, 2012). A doe in estrus is restrained in a neck clamp and the buck is allowed to mount her. Immediately when the buck mounts a doe, the penis is directed into the AV for ejaculation to take place in the collection glass.

2.4.1.1. Semen extenders

The main purposes of a cryopreservation extender are to supply the sperm cells with a source of energy, protect the cells from cold shock and maintain a conducive environment for the spermatozoa to survive temporarily. In general, a goat sperm cryopreservation medium includes a non-penetrating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant (glycerol, ethylene glycol, or dimethyl sulfoxide DMSO), a buffer (Tris or Test), one or more sugars (glucose, lactose, raffinose, saccharose, dextran or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Sharma, 2018). A non-fat dried skim milk diluent (Corteel, 1974) or a Tris–glucose diluent (Sharma et al., 2020; Beltran et al., 2013) is most commonly used for cryopreserving goat sperm. Various researchers suggested Tris-citric acid as the most satisfactory buffer for goat spermatozoa (Sharma et al., 2020; Mishra et al., 2010). Recently, coconut based extenders (Mollineau et al., 2011; Waidi et al., 2007), soybean based extenders (Kakati et al., 2019; Roof et al., 2012), and plant extract extenders (Zaenuri et al., 2014; Zanganeh et al., 2013) have also been used with good results.

2.4.1.2. Role of egg-yolk

Egg-yolk is a main component of semen extenders and has been used since 1939 in bull semen and still remains popular (Amirat et al., 2004) as it has excellent sperm cell protection (Celeghini et al., 2008) for cryopreservation of most mammalian species, including bulls, rams, and goats (Moussa et al., 2002). It is a nonpenetrating cryoprotective component and is globally used for sperm preservation. Chicken egg yolk consists of phospholipid, cholesterol and low-density lipoprotein. These are protective components that protect the membrane phospholipid integrity during cryopreservation (Aurich, 2005; Amirat et al., 2004) and prevent shock during cooling and freezing, which improves viability (Salamon and Maxwell, 2000) and activity (Beran et al., 2012) of spermatozoa. The concentration of egg yolk in extenders depends on the composition of extenders and also on the processes applied for preservation of semen. A wide range of egg yolk concentrations have been examined in extenders for the preservation of buck semen. Ranjan et al. (2015) reported that 10% egg yolk gives better protection to sperm than 15% and 20% egg yolk while adding in tris-based extender for buck semen preservation. But progressive motility

and live sperm were better when Black Bengal buck semen was extended with 2.5% egg yolk tris buffer than with 5%, 7.5% and 10% egg yolk for preservation.

2.4.1.3. Role of skimmed milk

Heated skimmed milk or whole milk is used as a medium in which sperm is diluted immediately and stored at 4°C or frozen in the presence of glycerol for sperm preservation (Kakar and Ganguli, 1978). Since skimmed milk contains almost zero lipids (< 0.1%, predominantly triglycerides) and is just as effective as whole milk in safeguarding sperm during storage at 4°C or cryopreservation (Foote et al., 2002; Almquist, 1954), lipids do not seem to be the constituent responsible for the protection afforded by milk. Lactose, which is found in cow milk at a concentration of 4.8 percent (w/v) (Amiot et al., 2002), can help extenders dilute sperm more effectively, but it is insufficient to protect sperm during storage at 4°C or cryopreservation (Choong and Wales, 1963; Garcia and Graham, 1987). The protective component of milk is most likely micelles of caseins, which are the key proteins of milk. Caseins (α, β and κ) are found in milk at ∼27 g/L of total milk proteins and exist as heterogeneous colloidal particles, named casein micelles (Amiot et la., 2002). Basically, casein micelles are made up of a hydrophobic core of α and β caseins covered by κ caseins 9 Dalgleish , 1998). Casein micelles extracted from milk have been proven to protect stallion, goat, ram, and bull sperm during storage at 4–5°C (O'shea and Wales, 1966; Batellier et al., 1997; Leboeuf et al., 2003). In the presence of glycerol, casein micelles can also protect bull sperm during freezing (Choong and Wales, 1963). The process by which casein micelles protect sperm during storage, on the other hand, remains unknown. Other proteins called whey proteins are soluble in milk (∼3.5 g/L) and include -lactalbumin, -lactoglobulin, albumin, and lactoferrin (Amiot et al., 2002). However, it is unclear if whey proteins have any protective effect on sperm during storage. Nor-Ashikin et al. (2011) reported D-glucose supplimented skimmed milk gives better result while preserving buck semen and found satisfactory post preserved quality of semen (Olurode and Ajala, 2016). But very limited information available in case of JP buck semen preservation by skimmed milk based extenders.

2.4.1.4. Short time preservation or chilled semen

Preservation of semen is the prime requirement for artificial insemination (AI) in goat. Chilled semen could be a valuable alternative to frozen semen with some beneficial point of view. It could be maintained higher percentages of motile sperm in a AI dose compared with frozen thawed semen at the same concentration. There is no any hazard related to liquid nitrogen management, semen could be used even if they are bad freezer (Vera-Munoz et al., 2011). It is well known that pregnancy rate combatively higher in liquid semen than frozen buck semen (Mara et al., 2007; Kharche et al., 2013) and cheap for preservation.

2.4.1.5. Long time preservation or cryopreservation

Cryopreservation of mammalian sperm is a complicated technique that requires careful consideration of a number of variables in order to get satisfactory results. To insure even minimal results, not only suitable diluents, sperm dilution rate, cooling rate and thawing rate are required, but also an intricate knowledge of the sperm physiology for the species is essential to optimize post-thaw recovery of sperm and consequently the fertility. Even though there are many similarities between goat sperm and sperm from other domestic species, such as the use of similar types of cryopreservation media, cryoprotectants, and freezing and thawing rates to cryopreserve these sperm, goat sperm require special attention to maximize their post-thawing. For example, there is a detrimental interaction between egg yolk, skimmed milk, and the secretions of the bulbourethral gland (phospholipase A, BUSgp60) in goat sperm that does not present in other species like the bull, boar, or ram (Pellicer-Rubio et al., 1997; Pellicer, 1995). However, various safe techniques have been studied to reduce these effects for buck semen preservation (Tabarez et al., 2017; Dorado et al., 2010). So, it could be good news for Jamunapari buck semen for cryopreservation with different extenders. Moreover, frozen semen is most useful in AI compare to other storage semen because of frozen semen can be stored for unlimited period and easily transport to any area.

2.5. Cervical artificial insemination (CAI)

Artificial insemination of goats is generally very similar to that of sheep. Insemination is carried out in natural oestrus 18 hours after onset of oestrus and the insemination dose contains minimum 240 × 106 spermatozoa (Paulenz, 2011). Among all AI techniques the cervical AI is the preferred option in small ruminants, when the external cervical os is visualized using illumination and the insemination pipette is then passed through the speculum into the cervix (without using excessive force) to a depth of 5 to 12 mm. After positioning the AI gun in the cervix, the insemination dose is deposited. Special attention should be paid to avoiding semen backflow or keeping it to a minimum. Cervical AI is combined with the use of fresh and chilled semen. The ideal time for AI is 55 hours after the removal of intravaginal progesterone inserts, or 15–17 hours after the onset of detected estrus. Originally, the use of frozen semen was limited because of the low kidding percentages obtained (25% to 35%) using cervical AI. This was associated with the reduced viability of frozen spermatozoa that resulted in low numbers of viable or undamaged spermatozoa reaching the fertilization site. However, in present time several developments in cervical AI skills, improved pregnancy rates are being achieved (Salamon and Maxwell, 2000). The inseminate volume and the number of motile spermatozoa required are 0.2 ml and min 200×10^6, respectively. Special attention has been paid to cervical AI with frozen semen in does (Fair et al., 2005; Paulenz, 2011). In the doe, cervical AI is the widely practiced method.

2.6. Pregnancy diagnosis

There are several methods available for the diagnosis of pregnancy in goats, which include non-return to estrus, recto-abdominal palpation, vaginal biopsy, abdominal palpation estimation of progesterone, oestronesulphate estimation, bimanual palpation technique, radiography, and ultrasonography.

Several authors have reported the higher accuracy rate of ultrasound scanning for early pregnancy diagnosis in small ruminants (Amer, 2010). For the past two decades, mainly three types of ultrasound systems (A-mode, Real-time B-mode, and Doppler) have been commonly in use for pregnancy diagnosis in sheep and goats. However, real-time B-mode ultrasound is found to be an accurate, rapid, practical, and safe tool

for pregnancy diagnosis, determination of fetal numbers, estimation of gestational age, and detection of fetal sex in small ruminants (Karen et al., 2001).

2.7. Pregnancy and fertility rate after AI with different types of semen

As AI is an established process for breeding, the fertility rate could be varying with methods applied for insemination and using different types of extended semen. Using chilled Sarda buck semen extended with skimmed milk-based extender, Mara et al. (2007) found PR 71.4 % in estrus synchronized goats where as Siqueira et al., found the PR 49.1% in Toggenburg goat estrus was synchronized with PGF2 alpha using 2.5% Tris-frutose-egg yolk chilled semen. Due to complex seminal attributes in buck semen for preservation the motility and viability of frozen semen is low (Muino-Blanco et al., 2008). Ranjan et al. (2020) observed 53.12±2.40% PR in Barbari goat using frozen thawed semem extended with tris based extenders where as Dorado et al. (2007) found only 42.90% PR in goats. From this point of view, the pregnancy and fertility rate by using preserved Jamunapari buck should be studied.

Chapter-3
Materials and methods

3.1. Description of the study area

Chattogram is considered the second-largest financial capacity city in Bangladesh, located at 22°20'18.24" N and 91°49'54.05" E. The topical monsoon climatic conditions are characterized by an annual average temperature of 13 to 32º C, humidity of 70-80%, and rainfall of 5.6 mm to 725.0 mm (Khan et al., 2019). In this metropolitan city, the goat population is 30,320 (DLS, 2017).

3.2. Animal selection and management

Two Jamunapari bucks (buck 1= 16 months and buck 2= 11 months) were used for semen collection and one doe (18 months) was used for dummy were chosen on the basis of phenotypic criteria and brought from a local goat owner. Animals were dewormed with an anthelmintic (Fenazol®, ACME Laboratories Ltd. Bangladesh) and vaccinated against Peste des petits ruminants (PPR) (PPR® Vaccine, LRI, Bangladesh) after acclimation. In the morning, the bucks were given 450g of commercial concentrate in mash form (crude protein content: 120g/kg DM and energy content: 10.4 MJ ME/kg DM), 350 g of grain, and 1.5 kg of green grass (Napier and/or German). In the afternoon, they were given a daily allowance of 500g of commercial concentrate and 1.5 kg of green grass per head. Ad libitum access to clean, safe water was provided. For 1-2 hours each day, the bucks were allowed to graze and exercise. Does were raised in a semi-intensive system. They were fed free grazing on pasture with the addition of a small amount of concentrate (300g/head in the morning and 400g/head in the evening). All animals were kept in a research goat shed near the Theriogenology Laboratory of the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU, Chattogram, Bangladesh (Figure 1).

Figure 1. Management of animals

3.3. Preparation of semen extenders

Tris citrate egg yolk (TCEY) and skim milk D-glucose (SD) based extenders were used in this study. Three types of locally manufactured extender were used. Tris citrate egg yolk (TCEY) based extender was prepared using 3.61g Tris (GPR®, BDH Laboratory Ltd., and England), 2.0g citric acid (Emprove®, Merck Ltd., and Germany), 0.5g fructose (D- Fructose®, Merck Ltd., India), deionized water to make 100 ml solution as stock and stored at 4°C temperature for a maximum two weeks (Rekha et al., 2016). For making the final extender on the day of semen collection; 2.5% (w/v) and 5% (w/v) egg yolk , 10000 IU of penicillin (Penicillin V®, Indobangla Pharmacuticals, Bangladesh) and 100 mg of streptomycin (Streptomycin®, Ranata Pharmacutical Ltd. Bangladesh) were added to the stock solution according to study design. After then, the extender was divided into Part-T1 (without glycerol) and Part-T2 (with 7% glycerol). Skim milk D-glucose (SD) extender was prepared by adding

10.0 g skim milk (Shape Up®, New Zealand Dairy Product Ltd. Bangladesh), 0.2 g D-glucose (D-glucose®, Merck Ltd., India) in 100 ml deionized water and heated at 91ºC for 10 min (Nor-Ashikin and Abdullah, 2011). This solution was cooled to room temperature and stored at 4°C temperature and used as a stock solution. On the day of semen collection, for making final extenders of SD, a similar protocol was applied which was used for preparing T extender. All the glycerol (7%, v/v) added parts of extenders such SDg, 2.5% TCEYg and 5% TCEYg were kept at the refrigerator (4°C) until use and parts of extenders without glycerol (SDw, 2.5% TCEYw and 5% TCEYw) were kept at 33-35°C in a water bath. For the chilling of semen, all these three types of extenders were prepared without providing glycerol accordingly and namely SDc, 2.5% TCEYc and 5% TCEYc**.** Extender was filtered by filter papers before use (WhatmanTM, cat No. 1001 125, 125 mm × 100 circles, GE Healthcare UK Limited, Amersham, China).

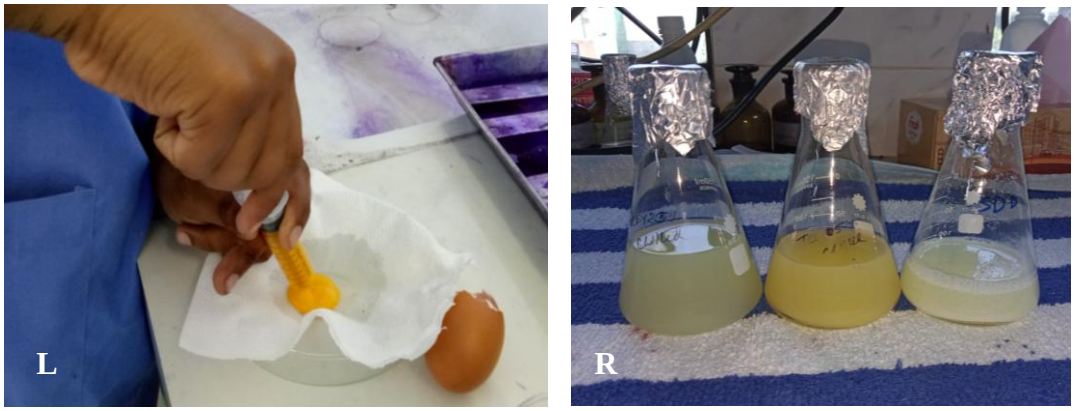


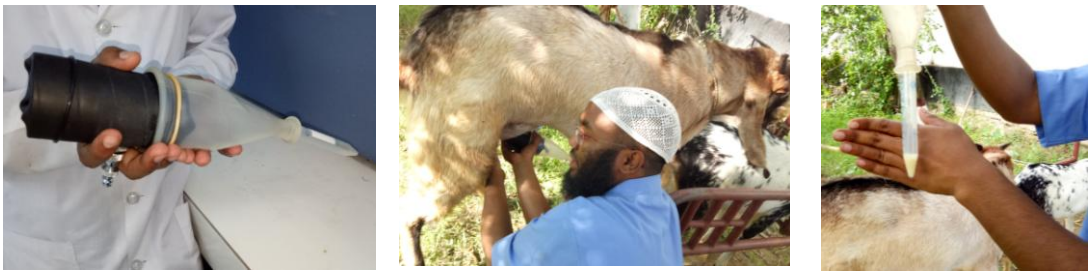
Figure 2. Egg yolk collection (L) and semen extenders (R)

3.4. Semen collection and evaluation

Before the study began, all bucks were trained to use a specific artificial vagina designed for sheep and goats (Minitube, Tiefenbach, USA). Semen was collected from two adult bucks by the Artificial Vagina (AV) method once a week (Figure 3). The water filling the AV at 51-52°C, and the internal temperuture was around 40-41°C (Hahn et al., 2019) All glassware for collection and handling were cleaned and sterilized using high pressure steam, dried and warmed at 35°C. A total of 62 ejaculates were collected from bucks during the monsoon season (October 2019 – June 2020). The collection was always performed in the morning (6:30 – 7:30 AM).

After collection, semen was kept at 33-35°C in a water bath until the fresh semen evaluation and media were added with it.

Macroscopic semen evaluation included all evaluations which can be performed with a naked eye such as semen color, volume and pH. The volume of semen was directly measured using the graduated collecting tube. pH paper (McolorpHast^{TM},Merck, Germany). Color was estimated by visual inspection and density was scored by making the tube slant with a range 1-5. The scoring system for semen density was as follows: 1: watery (400-1000 × 10^6 sperm/ml); 2: thin milky (1000-2500 × 10^6 sperm/ml); 3: thin creamy (2500 – 3500 × 10^6 sperm/ml); 4: creamy (3500 – 4500 × 10^6 sperm/ml); and 5: thick creamy consistency (4000 – 6000 × 10^6 sperm/ml) (Martin et al., 2012). A phase contrast microscope was used to do the microscopic investigation (Gallenhamp, No. 82TT8, Cat no. M/6-200-H-Hz60, England). Phase contrast microscope (Olympus, Japan) was used to observed morphology of spermatozoa under 40x magnifications. A drop of '20 µl' of semen was placed on a pre-warmed (37ºC) slide without a cover-slip and examined under a phase-contrast microscope '10x' to assess mass activity "wave motion." The mass activity was scored 0= no perceptible motion; 1=weak motion without forming any waves; 2=small/slow moving waves; 3=vigorous movement with moderately rapid waves and eddies and 4=dense, very rapidly moving waves and eddies (Shamsuddin et al., 2000). To evaluate sperm motility, 5 µl of diluted semen was placed on pre-warmed (37ºC) slide, covered with a cover slip and evaluated (40x). The concentration of spermatozoa was determined using a Neubauer counting chamber of haemocytometer (Marienfild® ,Germany) and the total numbers of spermatozoa were calculated by multiplying the volume of the ejaculate by the concentration according to Bane, 1952. (Figure 4).



AV set preparation Semen collection process from buck

Figure 3. Collection of Jamunapari buck semen

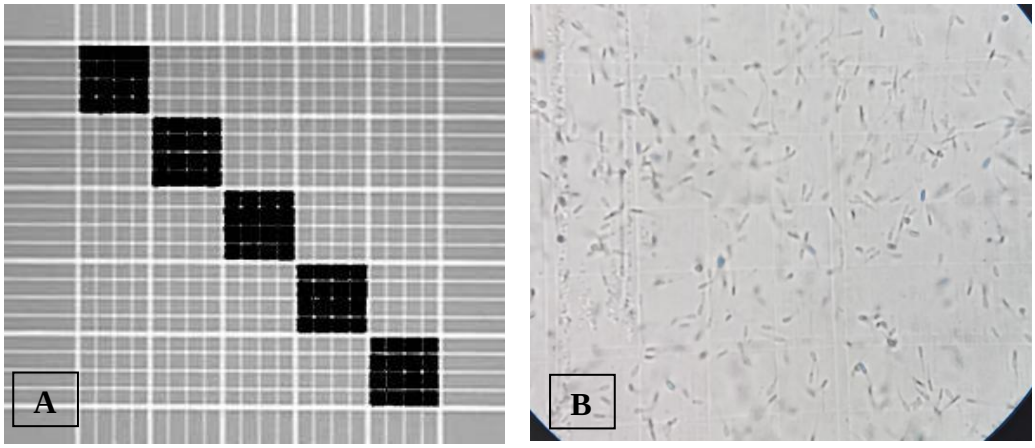


Figure 4. A- Counting area (Adapted from Azizunnesa, 2015); B- Neubauer counting chamber of haemocytometer with spermatozoa (1:200 dilution)

An Eosin-nigrosin stain was used to determine the viability of spermatozoa. One drop of semen and one drop of eosin-nigrosin stain were placed on a clean slide and mixed with a clean stick. A thin smear was made, dried in the air, and examined under (40x). At least 200 spermatozoa were examined from each smear to calculate the percentage of live spermatozoa (Figure 5). Sperm that were colorless were classified as live and those that had any pink or red coloration were considered dead, with the exception of sperm with a slight pink or red appearance restricted to the neck (leaky necks), which were assassinated as live (Mortimer,1994). A hypo-osmotic swelling test (HOST) was used to detect functionally integrated spermatozoa. The HOS solution was prepared by mixing 9 g of fructose and 4.9 g of sodium citrate into 1 L of deionized water. 20 µl of semen were mixed with 200 ml of HOS solution and incubated for 60 minutes at 37ºC. After incubation, 5µl of HOS mixed semen was placed onto a pre-warmed (37ºC) slide, covered with a cover slip and examined under a microscope (40x). Sperm with a curled tail was considered HOST positive. At least 200 spermatozoa were examined in different microscopic fields to calculate the percentage (Chetna et al., 2014; Revell and Mrode, 1994). Normal percentages of spermatozoa were evaluated according to normal head, acrosome, mid-piece, and tail length. Spermatozoa were counted using Rose-Bengal dye, where at least 200 spermatozoa were counted (Ax et al., 2000) (Figure 6).

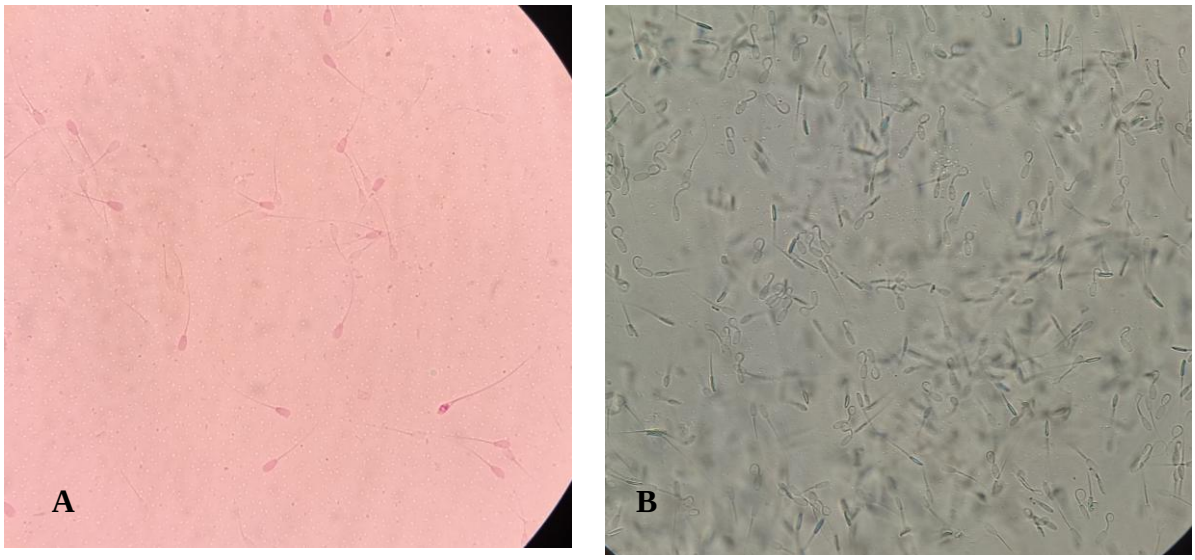


Figure 5. Viability (A) (40x) and HOST +ve spermatozoa (B) (40x)

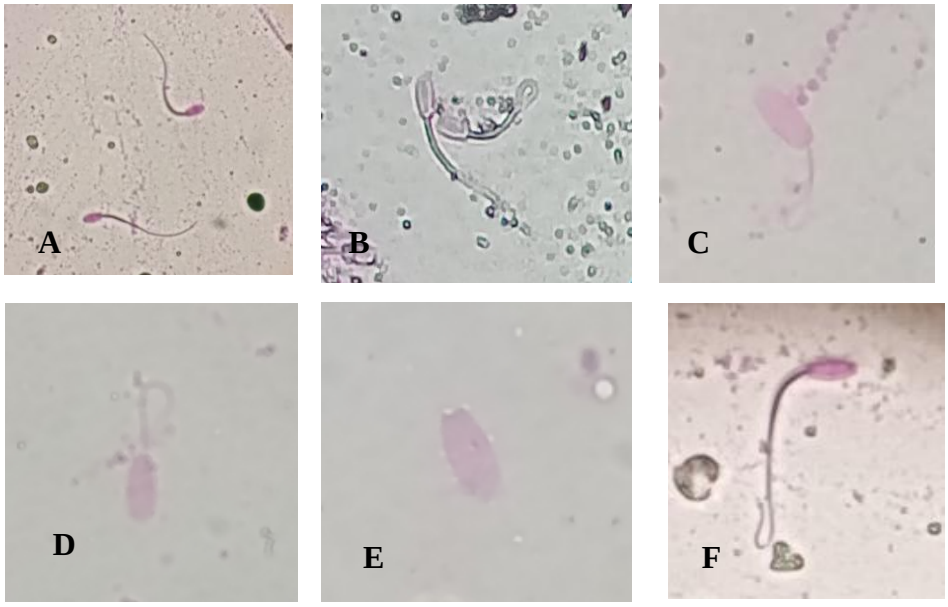


Figure 6. Normal abnormal spermatozoa (100x): A. Normal Sperm, B: Coiled tail, C. Short coiled tail, D. Bent tail, E. Detailed sperm, F. Curled tail

3.5. Semen preservation

Collected semen was preserved as chilled and frozen form. Each ejaculate from respective bucks was examined macroscopically and microscopically. Mass motility (0–5) and concentration (10^6/ml) of spermatozoa were evaluated. After the individual primary evaluation, all ejaculates from experimental bucks were transferred into one

sterile falcon tube and stored at 35º C as a pooled semen sample (Dorado et al., 2007). Then, that pooled semen was divided into two parts, one for chilling and the other one for cryopreservation of Jamunapari buck semen, and each part of fresh pooled semen was divided into another three fractions and kept in a separate eppendorf tube for chilling and freezing respectively.

3.5.1. Chill semen preservation

After preliminary evaluation of fresh semen, the required volume of extender was calculated, then diluted those fractions to previously made stock solutions (SDc, 2.5% TCEYc and 5% TCEYc), and kept at 4-5º C until evaluation (Figure 7). The chilled semen was held at room temperature for 4-5 minutes before evaluation to adjust the environmental temperature. On Days 1, 2, 3 and 4 the chilled semen was examined for individual motility, viability, functional integrity, and normal morphology.

3.5.2. Cryopreservation

For freezing, the required volume of semen extender was calculated and then three eppendorf containing fresh semen samples were diluted with the extender parts (without glycerol) SDw, 2.5% TCEYw and 5% TCEYw, separately at room temperature and kept at 4ºC for 2 hrs for equilibrium, then extender parts (with glycerol) such as SDg, 2.5% TCEYg and 5% TCEYg were added, respectively. Thereafter, the final mixed extended semen named SDf, 2.5% TCEYf and 5% TCEYf were loaded into a 0.25 ml semen straw (Minitube GmBH, Germany), sealed by the sealer machine, and held for the next 2 hrs at 4°C. After 4 hours of equilibration, all straws were exposed to liquid nitrogen vapor for 6 minutes. Then straws were transferred to liquid nitrogen (-196ºC) for storage until thawing (Figure 7). The final concentration of each frozen semen was 100 millions/ ml. Frozen semen straw was evaluated for individual motility, viability, functional integrity, and normal morphology on days 1, 5, 10 and 20 after cryopreservation.

Figure 7. Chilled (A) and Frozen semen preservation (B. Sealing of straw, C. Equilibration at 4ºC, D. Straw in Liq. N_2 vapor, E. Semen straw preserved in -196º C).

3.6. Artificial insemination

Cervical artificial insemination (CAI) was performed on an estrous does registered at S.A.Q. Teaching veterinary hospital, CVASU, by local farmers.

3.6.1. Semen processing before artificial insemination

Chilled semen (SDc, 2.5% TCEYc and 5% TCEYc) was warmed, evaluated, and loaded into 0.25 mL straws. Frozen semen (SDf, 2.5% TCEYf and 5% TCEYf) straw was thawed at 39–40 ºC for 13–14 seconds and motility was confirmed by direct microscopic examination. Then, semen straw was loaded into an artificial insemination gun (AI Gun®, Bal-Krishan Plasikrafts Ltd., India) prior to insemination.

3.6.2. Cervical artificial insemination (CAI)

Estrous does were restrained upright in a travis at 45º. The perineal region was cleaned with a fresh sterile gauge. A lubricated vaginal speculum was inserted into the vagina (Figure 8). The external os of cervix was visualized by using penlight (Figure 8-B). Semen straw loaded AI gun was introduced into the cervix and tried to push forward by moving the AI gun side to side and then forward to cervix (Figure 8-C). Semen was then deposited slowly into the cervix as deep as possible (Kharche et al., 2013). The does were held in the same position for 2–3 minutes and the clitoris was massaged gently.

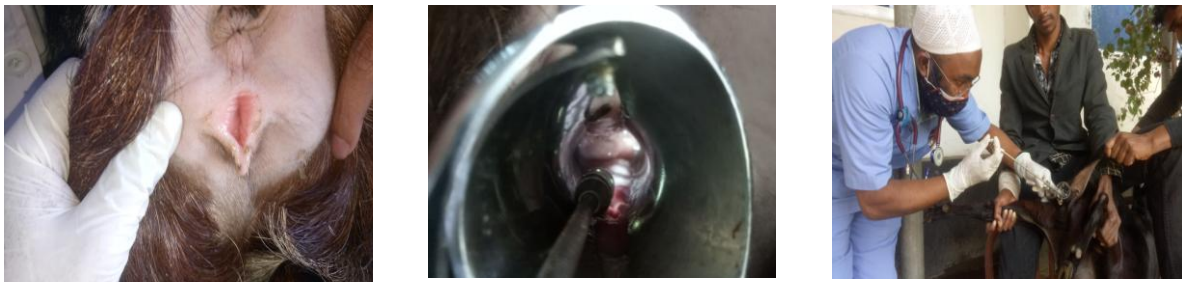


Figure 8. Estrus detection of doe (A); Observation by pen light (B) and Cervical AI (C)

3.7. Pregnancy detection

All inseminated does were subjected to ultrasonography scanning (Figure 9) after 2-3 months of insemination for the detection of fetus using a digital ultrasonic diagnostic imaging system with a semi-convex ultrasonic transducer 3.5–5 MHz (ECM Exago, France).

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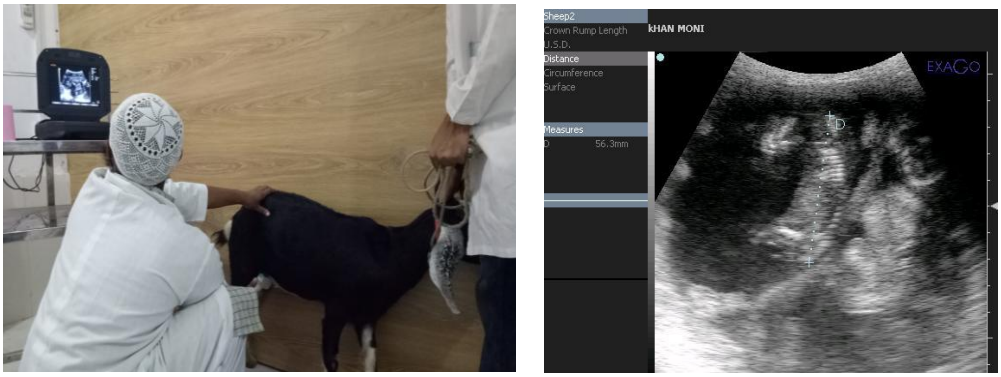

Figure 9. Pregnancy detection by ultrasonography scanning

Figure 10. Kids found after CAI in goat by studied Jamunapari buck preserved semen

3.8. Statistical analysis

Data on fresh, chilled, and frozen semen quality, cervical insemination, and pregnancy diagnosis were recorded. The collected data are expressed as mean ± SEM. All variables were checked for outliers and inconsistencies in Microsoft Excel 2007. An independent samples t-test was performed to compare semen parameters between two bucks' fresh semen and two egg yolk-sourced semen extenders. One-way ANOVA with Tukey's Post Hoc test was performed to evaluate the effect of extenders and different preservation times on the quality of Jamunapari buck semen. Pearson's chi-square test was performed to compare the PR between chilled and frozen semen extended with different extenders. All statistical analyses were performed using STATA version 13.0 for Windows (Stata Corp. College Station, USA). Statistical significance was set at $p \leq 0.05$.

Chapter- 4

Results

4.1. Fresh semen quality of Jamunapari buck

In Bangladesh, there is little information on the various fresh semen parameters of jamunapari (JP) bucks. The color of the fresh semen was creamy in buck 1 and creamy white in buck 2. The volume, concentration of spermatozoa, sperm viability, and HOST +ve sperm were higher in Buck 1 than in Buck 2 ($p<0.05$). However, there was no difference ($p \geq 0.05$) in seminal pH, density and mass motility between the two bucks (Table 1).

Table 1: Fresh semen quality of two different Jamunapari bucks (n=62)

Semen parameters	Buck 1	Buck 2	p value
Volume (ml)	1.33±0.10	1.06±0.15	0.03
Color	Creamy	Creamy white	---
pH	6.63±0.17	6.58±0.13	0.45
Density (0-5 score)	4.19±0.06	4.05±.07	0.13
Mass motility (0-5 score)	4.13±0.07	4.26±0.06	0.19
Conc. of spermatozoa (10^6/ml)	3166.34±22.31	2908.42±41.03	<0.001
Sperm viability (%)	85.06±0.21	82.73±.73	<0.05
Normal sperm (%)	94.73±0.20	93.91±0.29	0.024
HOST +ve sperm (%)	82.53±0.18	80.10±0.47	<0.05

Values are mean±SEM

4.2. Effect of different extenders and preservation time on quality of chilled semen

The mean concentration of spermatozoa of Jamunapari buck pooled fresh semen sample's was $3017.51\pm31.53\times10^6$ per ml.

Table 2 represents the effect of different extenders on the spermiogram of chilled semen with a preservation time. The proportion of motile, viable, functional integrity of spermatozoa and normal morphology of sperm was higher in samples extended

with 5% TCEYc than in the other two extenders in respect of preservation time ($p<0.001$ for all).

Table 2: Effect of different extenders on quality of chilled semen within preservation time (n=62)

Parameters (%)	Preservation time	Extenders		
		SDc	2.5 % TCEYc	5 % TCEYc
Sperm motility	Day 1	$71.20^{c}\pm0.4$	$76.95^{b}\pm0.3$	$79.60^{a}\pm0.4$
	Day 2	$68.51^{c}\pm0.3$	$71.40^{b}\pm0.4$	$73.08^{a}\pm0.4$
	Day 3	$63.81^{c}\pm0.2$	$67.12^{b}\pm0.4$	$69.01^{a}\pm0.4$
	Day 4	$60.35^{c}\pm0.6$	$62.37^{b}\pm0.3$	$64.30^{a}\pm0.5$
Sperm viability	Day 1	$77.15^{c}\pm0.4$	$80.28^{b}\pm0.2$	$82.50^{a}\pm0.6$
	Day 2	$72.62^{c}\pm0.4$	$76.41^{b}\pm0.5$	$78.17^{a}\pm0.3$
	Day 3	$65.37^{c}\pm0.3$	$70.81^{b}\pm0.1$	$73.31^{a}\pm0.2$
	Day 4	$61.08^{c}\pm0.4$	$67.93^{b}\pm0.3$	$69.20^{a}\pm0.1$
Normal sperm	Day 1	$89.78^{c}\pm0.4$	$90.21^{b}\pm0.3$	$92.85^{a}\pm0.2$
	Day 2	$85.79^{b}\pm0.5$	$87.80^{a}\pm0.3$	$88.21^{a}\pm0.2$
	Day 3	$82.16^{c}\pm0.3$	$83.67^{b}\pm0.2$	$85.48^{a}\pm0.3$
	Day 4	$77.85^{c}\pm0.4$	$80.02^{b}\pm0.2$	$82.28^{a}\pm0.4$
HOST+ve Sperm	Day 1	$76.43^{b}\pm0.5$	$79.95^{a}\pm0.7$	$80.31^{a}\pm0.4$
	Day 2	$71.88^{c}\pm0.6$	$75.13^{b}\pm0.4$	$76.24^{a}\pm0.4$
	Day 3	$66.12^{c}\pm0.3$	$70.19^{b}\pm0.6$	$72.62^{a}\pm0.5$
	Day 4	$60.47^{c}\pm0.3$	$65.18^{b}\pm0.3$	$69.05^{a}\pm0.6$

Values are mean±SEM, different superscript letters a,b,c indicate significant difference ($p<0.05$) between different extenders within preservation time.

Figures 11.a-11.d (Appendix 1) represents the effect of preservation time on chilled semen quality among different extenders. The preservation time had a significant effect on the quality of semen for each extender where increased preservation time reduced sperm quality ($p<0.001$).

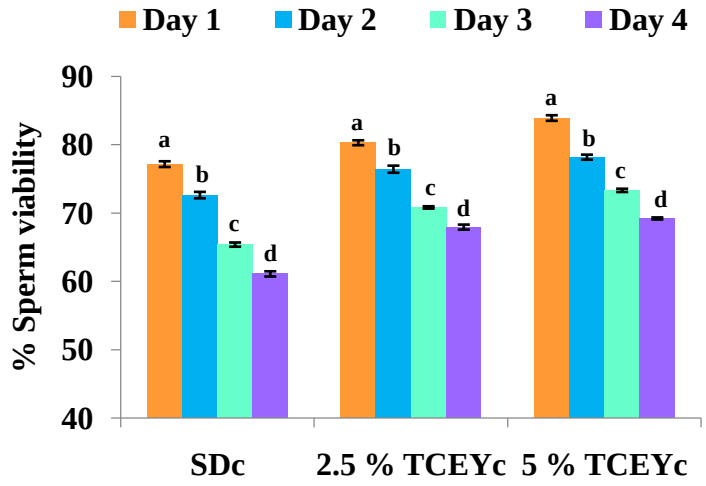

Figure 11.a. Effect of preservation time on Sperm viability in different extenders. Superscripts a,b,c,d indicates significant difference among preservation time ($p<0.05$)

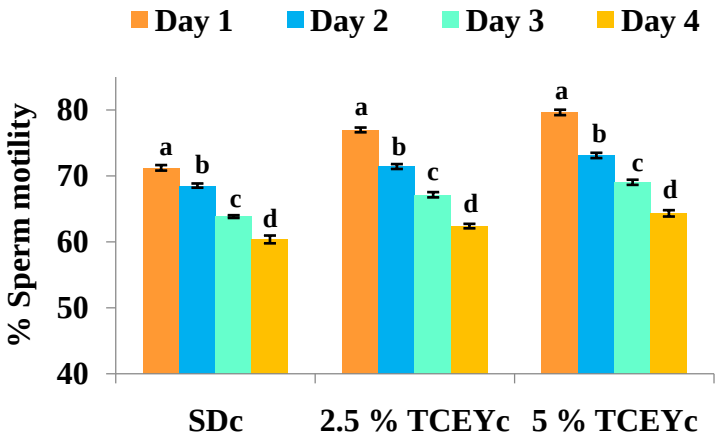

Figure 11.b.: Effect of preservation time on Sperm motility in different extenders. Superscripts a,b,c,d indicates significant difference among preservation time ($p<0.05$)

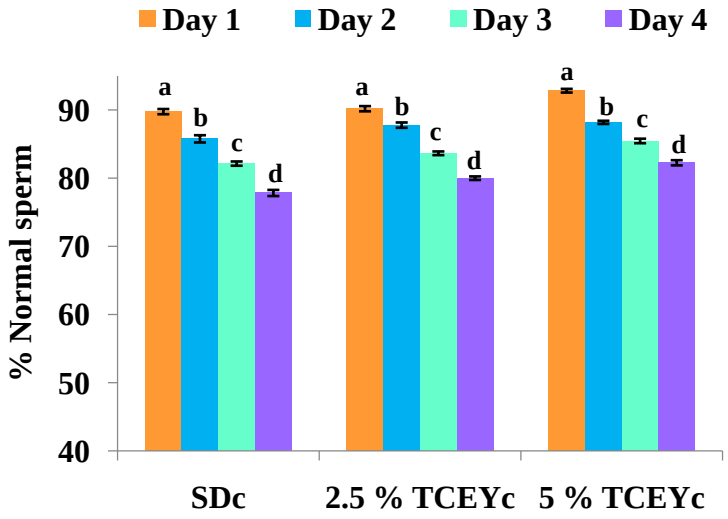

Figure 11.c.: Effect of preservation time on normal sperm in different extenders. Superscripts a,b,c,d indicates significant difference among preservation time ($p<0.05$)

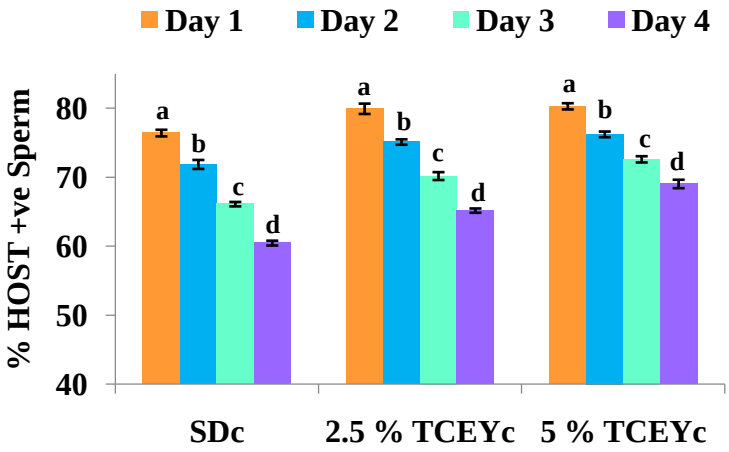

Figure 11.d. Effect of preservation time on HOST+ve sperm in different extenders. Superscripts a,b,c,d indicates significant difference among preservation time ($p<0.05$)

4.3. Effect of different extenders and cryopreservation time on post thawed quality of frozen semen

The effect of different extenders on frozen-thawed semen quality are presented in Table 3. Among different extenders, the 5 % TCEYf maintained higher motility, viability, functional integrity and normal morphology of spermatozoa compared with 2.5 % TCEYf and SDf , respectively with different cryopreservation time ($p<0.001$ for all) (Table 3).

Table 3: Effect of different extenders on quality of frozen-thawed semen within cryopreservation time (n=62)

Parameters (%)	**Cryopresvation time**	**Extenders**		
		SDf	**2.5 % TCEYf**	**5 % TCEYf**
Sperm motility	Day 1	$49.6^{c}\pm0.3$	$55.8^{b}\pm0.6$	$61.42^{a}\pm0.2$
	Day 5	$49.35^{c}\pm0.6$	$55.51^{b}\pm0.4$	$61.14^{a}\pm0.5$
	Day 10	$49.01^{c}\pm0.4$	$55.29^{b}\pm0.7$	$60.97^{a}\pm0.3$
	Day 20	$48.93^{c}\pm0.4$	$54.97^{b}\pm0.3$	$60.92^{a}\pm0.6$
Sperm viability	Day 1	$56.34^{c}\pm0.5$	$62.54^{b}\pm0.4$	$66.89^{a}\pm0.3$
	Day 5	$56.08^{c}\pm0.5$	$62.15^{b}\pm0.3$	$66.67^{a}\pm0.3$
	Day 10	$55.91^{c}\pm0.6$	$61.84^{b}\pm0.3$	$66.33^{a}\pm0.3$
	Day 20	$55.73^{c}\pm0.3$	$61.81^{b}\pm0.3$	$66.05^{a}\pm0.4$
Normal sperm	Day 1	$78.53^{c}\pm0.4$	$82.65^{b}\pm0.2$	$84.12^{a}\pm0.3$
	Day 5	$78.26^{b}\pm0.4$	$82.34^{a}\pm0.4$	$83.92^{a}\pm0.2$
	Day 10	$77.93^{c}\pm0.3$	$82.05^{b}\pm0.3$	$83.61^{a}\pm0.1$
	Day 20	$77.71^{c}\pm0.5$	$81.83^{b}\pm0.2$	$83.28^{a}\pm0.2$
HOST+ve Sperm	Day 1	$53.15^{c}\pm0.4$	$60.03^{b}\pm0.3$	$64.18^{a}\pm0.2$
	Day 5	$52.85^{c}\pm0.2$	$59.8^{b}\pm0.2$	$63.85^{a}\pm0.3$
	Day 10	$52.64^{c}\pm0.1$	$59.52^{b}\pm0.3$	$63.79^{a}\pm0.1$
	Day 20	$52.43^{c}\pm0.5$	$59.26^{b}\pm0.2$	$63.37^{a}\pm0.2$

Values are mean±SEM, different superscript letters a,b,c indicate significant difference ($p<0.05$) between different extenders within cryopreservation time.

Figures 12.a- 12.d (Appendix 2) represented the effect of preservation time on post thawed frozen semen quality among different extenders. Preservation time did not affect motility, viability, functional integrity, and normal morphology of spermatozoa for each semen extender for JP buck semen cryopreservation ($p > 0.05$).

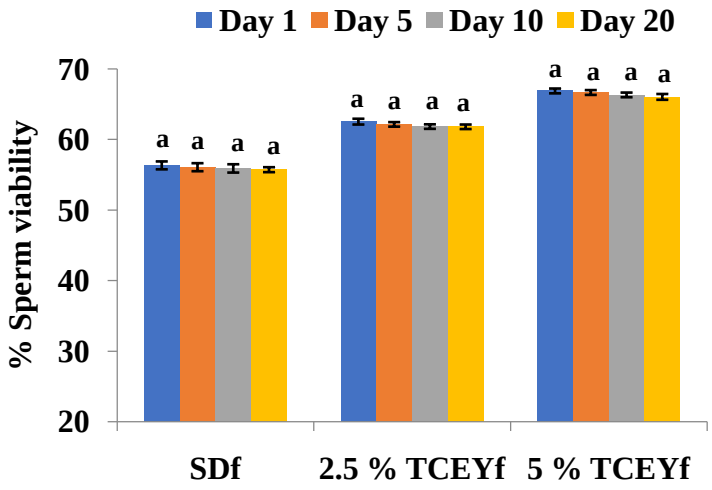

Figure 12.a. Effect of cryopreservation time on sperm viability in different extenders. Superscripts a,a indicates non-significant difference among cryopreservation time ($p>0.05$)

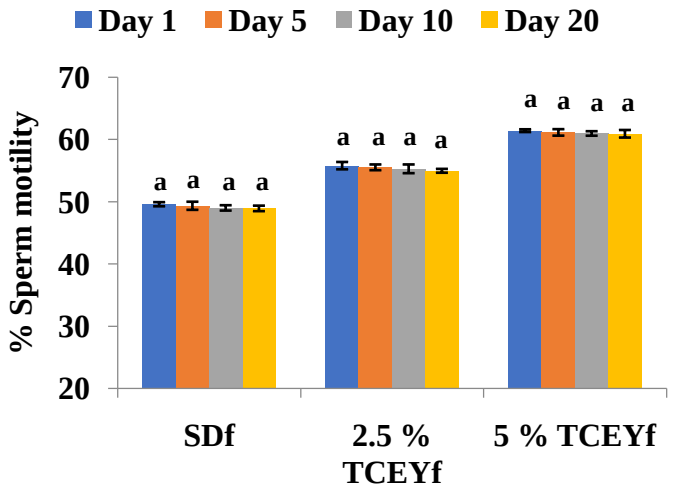

Figure 12.b. Effect of cryopreservation time on sperm motility in different extenders. Superscripts a,a indicates non-significant difference among cryopreservation time ($p>0.05$)

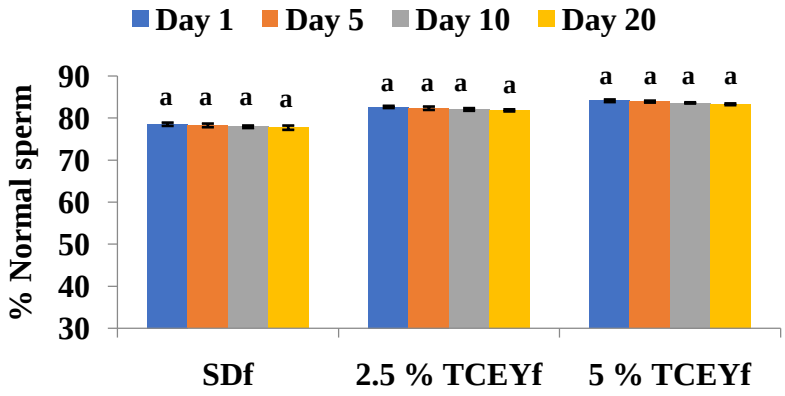

Figure 12.c.: Effect of cryopreservation time on normal sperm in different extenders. Superscripts a,a indicates non-significant difference among cryopreservation time ($p>0.05$)

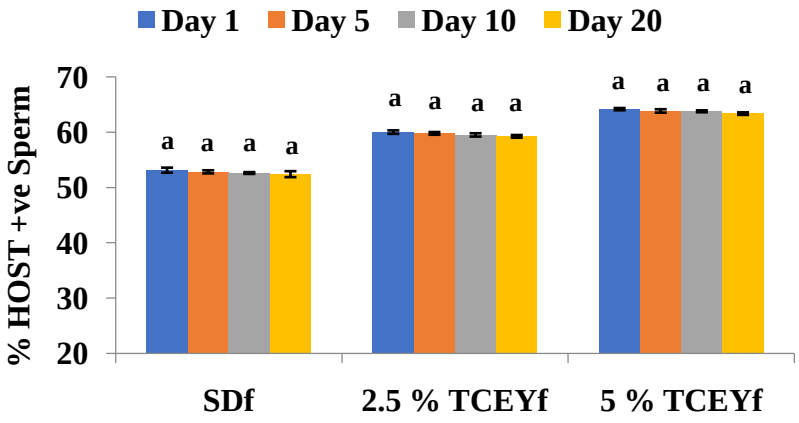

Figure 12.d. Effect of cryopreservation time on HOST+ve sperm in different extenders. Superscripts a,a indicates non-significant difference among cryopreservation time ($p>0.05$)

4.4. Evaluation of Jamunapari buck semen fertility

The fertility was assessed by the evaluation of pregnancy rate (PR) following CAI using chilled and frozen-thawed semen extended with different semen extenders, which is presented in Table 1 and Table 2 with different semen extenders.

Chilled semen extended with 2.5% TCEYc, 5% TCEYc and SDc had no significant effect ($p>0.05$) on fertility after CAI in goats (Table 4). However, between two egg yolks containing extenders, the PR was significantly higher in 5% TCEYc (61.11%) than in 2.5% TCEYc (42.10%) ($p<0.05$).

Table 4: Fertility of chilled Jamunapari buck semen extended with different semen extenders after CAI in goats

Type of Semen	Inseminated doe	Pregnant doe	Pregnancy Rate (PR %)	p value	Overall PR (%)
2.5% TCEYc	19	8	42.10	0.12	49.12
5% TCEYc	18	11	61.11*		
SDc	20	9	45.00		

* Superscript (*) indicate no significant difference ($p > 0.05$) within column in respect of extenders.

In case of frozen thawed semen extended with 2.5% TCEYf, 5% TCEYf and SDf, the PR following CAI varied significantly ($p < 0.05$) (Table 5).

Table 5: Fertility of frozen-thawed Jamunapari buck semen with different semen extenders after CAI in goats

Type of Semen	Inseminated doe	Pregnant doe	Pregnancy Rate (PR %)	p value	Overall PR (%)
2.5% TCEYf	28	9	32.14^{b}	0.04	35.36
5% TCEYf	28	13	46.42^{a}		
SDf	26	7	26.92^{c}		

* Superscript (^{a,b,c}) indicate significant difference ($p < 0.05$) within column in respect of extenders.

Chapter-5

Discussion

There is a lack of information on fresh semen spermiograms, semen preservation, and the evaluation of the fertility of Jamunapari buck semen in Bangladesh. As a result, a comprehensive study was conducted to investigate the fresh semen quality, semen preservation and evaluation, the effect of storage time and different extenders on semen preservation, and the fertility of preserved Jamunapari buck semen following CAI in goats.

5.1. Fresh semen quality of Jamunapari buck

Fresh semen volume, concentration, motility, morphology, and functional integrity are all important characteristics that lead to good-quality semen preservation required for AI. In males, semen volume is an important factor for assessing semen and reproductive performance (Ax et al., 2000).In this study the jamunapari buck semen volume was varied from 1.16±0.15 ml to 1.33±0.10 ml which differed significantly ($p<0.05$) between the bucks. This result was slightly close consistent with Islam et al. (2019) who found 1.49±0.19 ml semen but is inconsistent with Kharche et al. (2013) who observed 0.54±0.04 ml semen from Jamunapari bucks. The concentration of spermatozoa per ml of semen was recorded 2908.42±41.03 to 3166.34±22.31 millions in this study, which was higher than that reported by Faruque et al. (2007), who obtained an average sperm concentration of 2828 ± 11.8×10^6/ml in Black Bengal buck. However, the present observation differed from that of Goswami et al (2020) who found 3356.73± 83.06 million/ml in Beetal bucks.

Viable spermatozoa of fresh semen varied from 82.73±.73 to 85.06±0.21%, which differed significantly between bucks. This finding resembles the same of Hossain (2007), who reported 84.99±0.38% to 85.6±20.57% viable spermatozoa in Black Bengal buck sperm. However, Kharche et al. (2013) found that viable spermatozoa in fresh semen in Jamunapari buck was 72.07±0.38%, which was lower than that in this study, and the variation may be due to alterations in genetic merits between the same breeds.

Functional integrity (HOST +ve) of spermatozoa of fresh semen in this study was a little less than Gojen et al. (2016) who found 85.37±0.85% in Black Bengal buck semen, but higher than the findings of Deori et al. (2018) in Assam Hill goat semen

(66.95 ± 0.74%). As there are no data available for the functional integrity of spermatozoa of Jamunapari buck fresh semen, the findings of this study varied from other findings, which could be due to breed variation in the same species (Berry et al., 2019).

The normal morphology of studied Jamunapari buck semen was slightly higher than the findings of Afroz (2005) and Afrin et al. (2014) who reported 91.07% and 90.59±1.18% normal sperm, respectively in Black Bengal buck semen. It is generally known that a higher percentage of normal spermatozoa in buck semen results in better fertility of the respective buck.

However, semen volume, sperm concentration, sperm viability, and sperm morphology were significantly greater in Buck 1 than in Buck 2 ($p<0.05$). This findings is in agreement with the result of Apu et al. (2008) seen in Black Bengal buck and Sharma and Sood (2021) seen in Gaddi and Chegu bucks .These differences in all parameters could be due to variations in age, maturity, general health condition, endocrine balance, process and frequency of semen collection, soundness of the sex organs, and genetic potential, as these affect the sexual glands and libido in bucks (Apu et al., 2008; Al-Ghalban et al., 2004; Salhab et al., 2003).

5.2. Effect of different extenders and preservation time on quality of chilled semen

The present study showed that the quality of chilled semen preserved with Tris-based extender was better than that of skimmed milk-based extender on days 1 to 4 of observation ($p<0.001$). Over the preservation period, Pawshe et al. (2017) and Shamsuddin et al. (2000) observed that the chilled semen quality of Tris-based extenders with egg yolk was far superior to that of semen extended from skimmed milk observed in Black Bengal and Malabari buck semen, respectively. This was attributed to the presence of a 60-kilodalton glycoprotein in the bulbourethral secretion with triglyceride lipase activity, which interacts with skim milk to create oleic acid from triolein, causing spermatozoa to deteriorate (Pellicer-Rubio et al., 1997), and cholesterol, which is a low-density lipoprotein present in egg yolk that preserves the integrity of spermatozoa (Aurich, 2005). Moreover, within the two Tris-based extenders, the 5% egg yolk containing extender maintained more good-quality preserved semen than the 2.5% egg yolk containing Tris-based extender after four

days of observation ($p<0.001$). These findings on sperm viability, motility, and functional integrity of chilled semen are similar to those of Anand et al. (2016), who found more viable, progressive motile and intact plasma membrane integrity of spermatozoa in 5% egg yolk containing Tris extender than in 2.5% egg yolk. Another study conducted by Shamsuddin et al. (2000) also supported the present findings that increasing egg yolk percentage in Tris-based extender gives better quality in Black Bengal buck semen in 4ºC, who used 2.5% EY and 1.5 % EY. Several studies have reported that the components of egg yolk have a lethal interaction with buck seminal plasma during buck semen preservation (Roy, 1957; Leboeuf et al., 2000). Egg yolk also has protective activity against spermatozoa in semen extenders (Anand et al., 2014). Anand et al. (2016) found that increasing egg yolk concentration increases the extent of lethal interaction while also improving protection against cold shock and cryoinjuries during semen preservation (Drobins et al., 1993). This could be the reason for the better quality of chilled semen between the two egg yolk percentages in the Tris-based extender for Jamunapari buck semen in this study. This indicates that the croprotective effect of egg yolk dominates over the lethal interactive losses during cold storage, with the result that the semen diluted with 5% egg yolk had greater values than diluted with 2.5 percent.

Eswaramohan et al. (2014) found that sperm viability decreased ($P<0.05$) in a time-dependent manner during chilling at 5–9°C in sodium citrate egg yolk extender and that sperm viability decreased from 87.74±0.22 to 77.57±0.24% in Jamuanapri buck semen within storage times (Day 1, 2, and 3), which were higher than present study values in tris- egg yolk based extenders but support the findings of this study regarding decrease of preserved semen quality over chilling time ($p>0.001$). This variation could be due to the variations in the composition of the extender. Moreover, the substantial decrease in sperm motility and viability during chilling could be due to the gradual depletion of nutrients, such as potassium, sodium, and plasma proteins (Udeh and Oghenesode, 2011). Anand et al. (2016) found semen viability, motility and functional integrity of spermatozoa while chilling of Barbari buck semen with 2.5 % and 5 % egg yolk containing Tris-based extender were (63.50 ± 1.09 vs 69.33 ± 1.65), (54.17 ± 1.56 vs 63.00 ± 1.65) and (60.83 ± 1.62 vs 64.50 ± 1.77), respectively, which is lower than this study results in four days of post-preservation. Age and breed of the same animal are important factors that affect both fresh and preserved semen quality (Lemma and Shemsu, 2015).

In the present study, the percentage of motile sperm significantly decreased during the preservation period ($p<0.001$) (Figure 11. b), which is in line with the findings of Olurode and Ajala (2016), who found sperm motility decreased 80.24±25.0% to 0% within 1–5 days of preservation at 5ºC by using goat skimmed milk. The significant decrease in sperm motility of stored liquid semen could be related to the gradual depletion of micronutrients and plasma proteins required for the high metabolic demands of sperm (Bergeron et al., 2007).

5.3. Effect of different extenders and cryopreservation time on post thawed quality of frozen semen

There are numerous advantages to freezing buck sperm for use in AI. It is critical to ensure the quality of frozen sperm as this has an impact on conception rates. There is limited data on the effects of different lab-made extenders on frozen buck semen. Unfortunately, no information is available in Bangladesh regarding the freezing of Jamunapari buck semen using skimmed milk and 2.5% and 5% TCEY extenders.

During storage at -196ºC, tris-based extenders (TCEY) maintained more spermatozoa with higher percentages of motility, viability, normal morphology, and functional integrity than skimmed milk (SD)-based extenders in this study ($p<0.001$).Similar results were reported by Dorado et al. (2007) with respect to the in vitro performance. The storage and quality of semen may vary depending on the extender used (Barbas and Mascarenhas, 2009; Salamon and Maxwell, 2000). Many parameters, such as freezing method, equilibration periods, cooling rate, and extenders, may influence spermatozoa function during freezing and thawing (D'alessandro et al., 2001). However, in caprine (Mishra et al., 2010), ovine (Quan et al., 2016) and bovine semen (Chaudhari et al., 2015), the beneficial effects of the TCEY extender have been extensively reported when compared with skimmed milk-based semen (Dorado et al., 2007).

The proportions of semen viability and motility differed significantly between the two extender sources in this study ($p<0.05$). This result is supported by that of Nor-Ashikin and Abdullah (2011), who found that skimmed milk D-glucose extenders had a lower ability to maintain high quality in cryopreserved Jermasia buck semen than tris-based extenders. This effect might be due to the presence of phospholipase A, a

specific enzyme found in goat sperm that interacts with the egg yolk and milk present in extenders to produce chemicals that degrade sperm quality (Pellicer-Rubio and Combarnous, 1998). These enzymes are thought to interact more extensively with milk during cryopreservation and hydrolyze the triglycerides of the sperm plasma membrane. Skimmed milk produces oleic acid, a fatty acid that is toxic to spermatozoa (Pellicer-Rubio et al., 1997), resulting in a significant decrease in sperm velocity. This impact could possibly be linked to the high calcium content in milk, which may be responsible for increasing the activity of phospholipase A, which requires calcium to function and hence increases the membrane's permeability to ions (Martins, 2006). However, Rahaman et al. (2018) revealed that skimmed milk provides better results for cryopreservation of indigenous ram semen than tris-fructose egg yolk-based extenders, which is contradictory to this study finding in Jamunapari bucks. This may be due to variations in the seminal composition of these species (Swelum et al., 2018).

This study showed that 5% egg yolk containing tris-based extender provides better frozen-thawed semen quality than 2.5% egg yolk in Jamunapari buck semen ($p<0.05$). Ranjan et al. (2015) discovered that increasing the egg yolk percentage in a tris-based extender by up to 15% improves the freezability of Jamunapari buck sperm. Lower concentrations of egg yolk were shown to be ineffective in protecting spermatozoa against freeze-thaw damage. The high egg yolk content in the extender may deactivate seminal plasma enzymes. Some components of egg yolk inhibit the enzyme lysophospholipase (Chauhan and Anand, 1990). When fresh semen was diluted (1:10), the ratio of phospholipase A2 to lysophospholipase decreased to 1/1.5. Several proteins, including β-lactoglobulin, transferrin, and albumin, have non-specific beneficial effects on the activity of bulbourethral lipase (Pellicer-Rubio and Combarnous, 1998). Furthermore, egg yolk hydrolysate toxicity is affected by pH, temperature, and the source of egg yolk (Leboeuf et al., 2000). The findings of Gojen et al. (2016) are contradictory to our findings, where they found that increasing the egg yolk percentage (2.5% to 10.0%) in tris-based extenders decreased the frozen semen quality in black bengal buck semen. Although the components of those tris-based extenders are similar and supplied an analogous buffering system during cryopreservation of those authors' findings, the obtained result of our study may differ from other findings due to the quantitative difference in the composition of the tris-

based buffer. Dorado et al. (2007) observed sperm motility (52.43±1.34%) and normal morphology of spermatozoa (72.217±2.01%) of adult Florida bucks in frozen-thawed semen in a skimmed milk-based extender, which was slightly lower than our findings in Jamunapari buck semen, which could be due to D-glucose supplementation in the skimmed milk extender and breed variation in this study.

As expected, the motility, viability, functional integrity, and normal morphology of frozen semen were unaffected by the preservation time ($p > 0.05$). Studies have revealed that freezing spermatozoa for an extended period does not affect their quality (Malik et al., 2015), despite the fact that there is an initial stage of degradation linked to the formation of ice crystals in the cell (Isachenko et al., 2003). After storage in liquid nitrogen for 1, 7, 15, or 30 d, the motility and viability of frozen-thawed alpaca sperm did not change (Bravo et al., 2013). According to this study, the motility, vitality, normal morphology, and functional integrity of frozen-thawed spermatozoa did not decrease over time. Thus, Jamunapri buck semen can be successfully frozen. However, the type of extender used can affect sperm quality.

5.4. Evaluation of fertility of Jamunapari buck on different semen

In this study, the semen quality of tris-based extenders was better after chilling than that of skimmed milk extenders; however, the pregnancy rate was not significantly different ($p > 0.05$). On the other hand, the 5% egg yolk containing tris-based extender had a higher pregnancy rate than the 2.5% egg yolk containing tris-based extender ($p<0.05$)

No studies have compared the effects of tris-based (TCEY) and skimmed milk (SD)-based chill semen extenders on the PR in goats. Furthermore, there is a lack of information available on jamunapri buck chilled semen diluted with these three extenders and used for CAI. Siqueira et al. (2009) found that the PR was 49.1% in Toggenburg goat estrus synchronized with PGF2 alpha using 2.5% Tris-frutose-egg yolk chilled semen, which was higher than that of the studied chilled semen extended with 2.5% TCEY (42.10%). Using chilled Sarda buck semen extended with a skimmed milk-based extender, Mara et al. (2007) found a PR of 71.4 % in estrus synchronized goats, which is greater than our result using chilled Jamunapari buck semen. It is well known that estrus synchronization increased reproductive

performance as well as conception rate in goats (Sen and Onder, 2016; Amarantidis et al., 2004).

The quality of frozen-thawed buck spermatozoa is low because they are more sensitive to cold shock than spermatozoa of other species (Muino-Blanco et al., 2008). The lower quality and complexity of the cervix and non-technical personnel for AI limits PR after CAI in goats (Ranjan et al., 2020).Therefore, CAI using frozen semen is not widely practiced in goats under field conditions (Arrebola et al., 2008). However, the fertility of goats using Jamunapari buck–frozen-thawed semen is an essential outcome. The conception rate was highly correlated with the depth of penetration by CAI. Although laparoscopic AI involving deposition of frozen-thawed semen directly into the uterus generally results 60–70% fertility (Salamon and Maxwell, 1995), the conception rate of cryo-preserved semen following cervical AI (CAI) is still very low at 16 – 40% (Kharche et al., 2013; Kumar and Naqvi, 2014). In goats, the Embrapa AI technique has produced satisfactory rates of cervical transposing and intrauterine AI, as well as reasonable pregnancy rates (Jeferson et al., 2017).

In Bangladesh, there is a lack of information about the fertility of goats inseminated with frozen semen from jamunapari bucks extended with different extenders. This study identified the effects of extenders used for frozen semen on PR after CAI in goats. The results showed significantly higher PR in naturally estrused goats using frozen-thawed semen extended with 5% TCEY extender than in the other two lab-made extenders ($p < 0.05$). Ranjan et al. (2020) observed 53.12±2.40% PR in Barbari goat using frozen thawed semem diluted with 10% EY containing tris fructose extender which is more higher than our result in goats inseminated with frozen thawed Jamunapari buck semen extended by 5 % and 2.5% TCEY (46.42 % vs 32.14%). Moreover, Kharche et al. (2013) found an overall RP of 53.13 % in goats using Jamunapari frozen-thawed semen, which is greater than our finding of overall PR (39.02 %) in a small number of inseminated does. Lower fertility after CAI in goats with frozen-thawed semen could be due to impaired transport of frozen-thawed sperm through the cervix (Martínez et al., 2012; Anand et al., 1989) and reduced viability of frozen-thawed spermatozoa (Ritar and Ball, 1993).

Dorado et al. (2007) reported that a tris-based extender results in better in vitro performance compared to milk, although these improvements were not reflected in fertility results. Semen doses cryopreserved in skimmed milk extender provided

greater pregnancy rates after CAI compared to those in tris-based extender (52.4% versus 42.9%)

The in vivo experiment did not confirm the in vitro superiority of the tris-based or skimmed milk extenders. Comparison of the resultant fertility showed inconsistent differences between the two extenders observed in vitro. This indicates that either tris-based or skimmed milk extender can be used for cryopreservation of goat semen for use in cervical insemination because both extenders maintain suitable viability of spermatozoa and fertilizing ability. To fully assess the extent to which the extender supported the fertilizing ability of the cryopreserved Jamunapri buck semen, a larger number of goats should be inseminated.

Limitations of the study

1. This study was hospital-based, and insemination of does was performed in the SAQ teaching veterinary hospital where an unknown stage of estrused does was brought for CAI by local farmers.
2. A small number of does were inseminated, which varied the fertility in respect of different extenders.
3. The age and physiological status of the experimental bucks were different, which may have affected the quality of fresh and preserved semen.

Chapter-6
Conclusions

Overall fresh semen quality varied significantly between Jamunapari bucks. Tris citric egg yolk (TCEY) extender provides better post-chilling and freezing quality of preserved Jamunapri buck semen than the skimmed milk extender (SD). The 5% egg yolk containing the tris-based extender was better than 2.5% for the chilling and freezing of Jamunapri buck semen, which indicates a slight increase in egg yolk in the TCEY extenders that enhances the quality of preserved buck semen. Fertility was significantly higher when CAI was performed with chilled and frozen semen extended with 5% TCEY than with 2.5% TCEY or SD. However, the overall PR in goats by using Jamunapari buck chilled and frozen semen extended with those three extenders was 49.12% and 35.36%, respectively.

Chapter-7

Recommendations and future perspectives

1. Jamunapari buck semen can be successfully preserved and used for artificial insemination in field condition with acceptable pregnancy rate.
2. Further study can be done to improve the semen extender for preservation of Jamunapari buck semen.

Chapter-8
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Appendix 1: Effect of preservation time on quality of chilled semen among extenders (n=62)

Parameters (%)	Semen extenders	Preservation time			
		Day 1	Day 2	Day 3	Day 4
Sperm motility	SDc	71.20^{a}±0.4	68.51^{b}±0.3	63.81^{c}±0.2	60.35^{d}±0.6
	2.5 % TCEYc	76.95^{a}±0.3	71.40^{b}±0.4	67.12^{c}±0.4	62.37^{d}±0.3
	5 % TCEYc	79.60^{a}±0.4	73.08^{b}±0.4	69.01^{c}±0.4	64.30^{d}±0.5
Sperm viability	SDc	77.15^{a}±0.4	72.62^{b}±0.4	65.37^{c}±0.3	61.08^{d}±0.4
	2.5 % TCEYc	80.28^{a}±0.2	76.41^{b}±0.5	70.81^{c}±0.1	67.93^{d}±0.3
	5 % TCEYc	83.90^{a}±0.6	78.17^{b}±0.3	73.31^{c}±0.2	69.20^{d}±0.1
Normal sperm	SDc	89.78^{a}±0.4	85.79^{b}±0.5	82.16^{c}±0.3	77.85^{d}±0.4
	2.5 % TCEYc	90.21^{a}±0.3	87.80^{b}±0.3	83.67^{c}±0.2	80.02^{d}±0.2
	5 % TCEYc	92.85^{a}±0.2	88.21^{b}±0.2	85.48^{c}±0.3	82.28^{d}±0.4
HOST+ve sperm	SDc	76.43^{a}±0.5	71.88^{b}±0.6	66.12^{c}±0.3	60.47^{d}±0.3
	2.5 % TCEYc	79.95^{a}±0.7	75.13^{b}±0.4	70.19^{c}±0.6	65.18^{d}±0.3
	5 % TCEYc	80.31^{a}±0.4	76.24^{b}±0.4	72.62^{c}±0.5	69.05^{d}±0.6

Values are mean±SEM, different superscript letters a,b,c,d,e indicate significant difference ($p<0.05$) among preservation time with different extenders in same row.

Appendix 2: Effect of cryopreservation time on quality of frozen thawed semen among extenders (n=62)

Parameters (%)	Extenders	Cryopreservation time			
		Day 1	Day 5	Day 10	Day 20
Sperm motility	SDf	49.6[a]±0.3	49.35[a]±0.6	49.01[a]±0.4	48.93[a]±0.4
	2.5 % TCEYf	55.8[a]±0.6	55.53[a]±0.4	55.29[a]±0.7	54.97[a]±0.3
	5 % TCEYf	61.42[a]±0.2	61.14[a]±0.5	60.97[a]±0.3	60.92[a]±0.6
Sperm viability	SDf	56.34[a]±0.5	56.08[a]±0.5	55.91[a]±0.5	55.73[a]±0.3
	2.5 % TCEYf	62.54[a]±0.4	62.15[a]±0.3	61.84[a]±0.3	61.81[a]±0.3
	5 % TCEYf	66.89[a]±0.3	66.67[a]±0.3	66.33[a]±0.3	66.05[a]±0.4
Normal sperm	SDf	78.53[a]±0.4	78.26[a]±0.4	77.93[a]±0.3	77.71[a]±0.5
	2.5 % TCEYf	82.65[a]±0.2	82.34[a]±0.4	82.05[a]±0.3	81.83[a]±0.2
	5 % TCEYf	84.12[a]±0.3	83.92[a]±0.2	83.61[a]±0.1	83.28[a]±0.2
HOST+ve Sperm	SDf	53.15[a]±0.4	52.85[a]±0.3	52.64[a]±0.1	52.43[a]±0.5
	2.5 % TCEYf	60.03[a]±0.3	59.8[a]±0.2	59.52[a]±0.3	59.26[a]±0.2
	5 % TCEYf	64.18[a]±0.2	63.85[a]±0.3	63.79[a]±0.1	63.37[a]±0.3

Values are mean±SEM, different superscript letters a,a indicate non-significant difference ($p>0.05$) among cryopreservation time with different extenders in same row.

Biography

Md. Moktadir Billah Reza, the author of this manuscript, was born on March 29, 1995, in the Naogaon district of Bangladesh. He passed the Secondary School Certificate (SSC) in 2010 and the Higher Secondary School Certificate (HSC) in 2012. Afterwards, he obtained a Doctor of Veterinary Medicine (DVM) degree from Chattogram Veterinary and Animal Sciences University (CVASU), Chattogram, in 2018. He performed clinical training in veterinary clinical medicine at Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), India, and the University of Putra Malaysia (UPM), Malaysia, in 2018. He then joined the MS degree in Theriogenology in 2019 in the Department of Medicine and Surgery, Faculty of Veterinary Medicine, Chattogram Veterinary and Animal Sciences University. He has published seven scientific research articles at home and abroad in the fields of Theriogenology and Molecular Biology. The author also received an NST Fellowship for his MS research and worked as a research assistant in the establishment of a semen bank project funded by the UGC-CVASU.