# CHAPTER 1: INTRODUCTION

## 1.1. Background:

Livestock has been playing a major role in increasing the agro-economy of Bangladesh. Goats are a type of livestock animal that is mostly raised by middle-class and lower-class individuals as a reliable source of food and revenue (Sahlu and Goetsch, 2003). Production profiles and important performance sources of goats are like meat, milk, skin, hair, and dung. According to the Department of Livestock (DLS) report, the total goat population is being reared at about 269.45 lakh (DLS, 2023) in Bangladesh with Black Bengal, Jamunapari (JP), Tutapuri, Beetal, and cross breed mostly (Bhowmik et al., 2014). Among them, JP is more popular due to its fast and high body weight gain, good milk production as well as good fertility rate in farming conditions with good adaptability and increasing resistance against common diseases in Bangladesh (Amin et al., 2001; Bhowmik et al., 2014; Hussain et al., 2010). In order to boost the number of JP goats produced, easy breeding facilities must be made available. Goat output can be improved by obtaining prominent breeding bucks, which are hard to come by because of their expensive upbringing, and castrating them at 5–11 weeks of age to produce superior meat and skin. A superior breeding policy can help to pick superior parents, increasing the likelihood of obtaining higher genotypic and phenotypic offspring (Bhatia and Arora, 2005; Jabbar et al., 2010). Artificial Insemination (AI) techniques are necessary for selective breeding bucks in order to develop the species by the use of semen from males with high genetic merit (Roberts and Foote, 1989). To achieve that goal, well-preserved diluted semen with a better extender is required for AI and may transfer to different parts of the country to spread the superior genotypes by increasing the life span of the stored semen.

Unlike bulls, Bucks require an appropriate semen extender in order to preserve highly concentrated spermatozoa (Evans and Maxwell, 1990). Spermatozoa under cold circumstances lose their ability to fertilize over time (Shamsuddin et al., 2000) and after two to three days, their motility and morphology are damaged (Alam et al., 2005). Polyunsaturated fatty acids make up a large percentage of buck sperm cells (Mann et al., 1980). They are especially vulnerable to rapid peroxidative damage due to the production of harmful reactive oxygen species (ROS) such as superoxide, peroxide, and hydroxyl radicals, which harm sperm cells structurally and reduce motility (Lenzi et al., 2002; Asadpouretal, 2011). Although, the seminal plasma has an antioxidant system that reduces the effect of ROS and protects the cells not enough of spermatozoa to protect against the ROS (Alvarez and Storey, 1982).

Antioxidant supplementation on cryopreservation media seemed to improve the quality of semen against free radicals-induced damage (Sariozkan et al. 2009; Memon et al. 2011; Azawi and Hussein, 2013). Vitamin C (L-ascorbic acid, ascorbate) is naturally present to scavenge free radicals and decrease lipid peroxidation (Anane and Creppy, 2001). Glutathione is biosynthesized in the body from the amino acids e.g. cysteine, glutamic acid, and glycine that react with ROS directly and it is a co-factor for glutathione peroxidase (GSHPx) that catalyzes the reduction of toxic H2O2 and others hydroperoxides, protecting from oxidative stress (Bucak et al., 2008). Improvement of motility and increased preservation time are gained in other species by adding GSH (Ansari et al., 2012; Ogata et al., 2015; Ahmad et al., 2021) and vitamin C (Hu et al., 2010; Achi et al., 2018) in chilled and frozen semen but there are no available data for goats. Therefore, the aim of this study was to test the hypothesis that different levels of vitamin C or glutathione might effectively protect buck semen from oxidative damage during cryopreservation in TCEY (tris citrate egg yolk) extender resulting in higher post-thaw sperm viability, motility, and fertility.

## 1.2. Objectives of this study:

The overall objectives of this study are taken through the following objectives:

1. Comparative evaluation of fresh semen between Jamunapari and Black Bengal bucks.
2. To evaluate the effects of different concentration of Glutathione and Vitamin C in preservation of Jamunapari buck semen.
3. Selection of better antioxidant supplementation for preservation of Jamunapari buck semen.

## 1.3. Hypothesis of this study:

It is hypothesized that, including antioxidants supplement e.g. glutathione and vitamin-C in the semen extender of Jamunapari buck semen may improve the semen quality parameters i.e. sperm motility, viability, morphology and functional integrity in both chilled and frozen semen.

**CHAPTER 2: REVIEW OF LITERATURE**

Goats are the first domesticated animals (Herre and Rohrs, 2001). It is the most important livestock species in Bangladesh which is called the “Poor man’s’’ cow and is raised by landless poor men, small farmers, and distressed women with little investment who live in geographically remote places and have few other options (Chowdhury et al., 2012). However, nowadays, goats are being reared by people from all classes due to their small size, sociable nature, high fertility rate, and ability to utilize a wide range of feed and fodder resources(Singh, 2003). It requires low investment but provides high remunerations in the form of multi-products such as meat, milk, fiber, skin, and manure.The subtropical monsoon climate, characterized by wide seasonal variations in rainfall, temperature, and humidity, is suitable for goat rearing in Bangladesh. However, to sustain this tradition and implement modern technology like semen preservation and artificial insemination, effective goat-rearing practices are needed in Bangladesh.

## 2.1. Jamunapari goat

There are many goat breeds in Bangladesh and Jamunpari (JP) is one of them. JP goats are multipurpose animals, producing meat, milk, skin, and hair (Amin et al., 2001). The goat breed originated from the Chakarnagar Region of Uttar Pradesh (U.P), India, and is known as Chambal Queen. The coat color of the JP goat is mainly white and white mixed light brown color is also found in Bangladesh. Buck's average weight is 50.70 kg and doe are 45.47 kg (Bhowmik et al., 2014). The sexual maturity of the JP males comes at the age of 9-12 months and females at 11-13 months (Hassan et al., 2010). Testis length, testis width, and scrotal circumference (SC) of JP buck were found as 17.3 cm, 11.5 cm, and 42.4 cm, respectively (Hassan et al., 2010). It has a comparatively bigger body size, tall, long legs (leggy), nose (parrot-like nose), large folded pendulous (hanging long) ears, and short and flat horns are major physical features. A thick growth of hair on the buttocks, known as feathers, obscures the udder when observed from behind. Its udder is well developed and round with large conical teats. Male goats that are being used for breeding purposes are called buck and the castrated male are called wither or khasi. On a farm, the buck is superior according to genetic impact because a buck can produce around 100 kids in a breeding season whereas does are incapable. Some seminal traits were found that volume, mass motility, motility, sperm concentration, live sperm% abnormal sperm percentage was 0.54±0.04, 3.58±0.14, 63.29±0.02, 3573.04±1.05x106, 72.07±0.04, 2.18±0.14 respectively by D Kharche et al., (2013). A buck's reproductive life span is shorter than it does. Scientific breeding programs are absent all over Bangladesh. People are using a traditional inbreeding program that decreases the genetic merits of goats. Very little information has been found about reproductive characteristics, semen preservation, and artificial inseminations of JP bucks to enhance genetic potentiality and production.

## 2.2. Black Bengal goat

Black Bengal goat (BBG) is the most common breed of goat in Bangladesh and it’s found all over the country (Mason 1969). The BBG is one of the most compliant, all-around adjusted, early maturing, prolific, productive, and tropical disease–resistant goat types of the world that produces incredible quality meat, milk, and skin. In Asia, 52.5% of the absolute goat population is on the earth, and Bangladesh has 60.1 million heads speaking to 68.3% of all-out ruminant animals (FAOSTAT, 2018). The male goat's weight is 20 kg and the doe goat of a similar age predicted 15 kg at 1 year old (Choudhury et al. 2012). Both genders have short, round, and hollow horns. More established bucks and does exhibit beards. Semen production of animals largely depends on several criteria such as age, maturity, nutritional status, health conditions, BCS, endocrine balance, and normality of sex organs (Peters, 2002). A breeding buck should have a minimum size scrotal circumference that correlates with sperm concentration per milliliter and total semen volume per ejaculate (Bongso et al., 1982). Lgboeli (1974) obtained the mean scrotal circumference (SC) of 20.9±0.3 cm for native Zambian bucks which is similar to the Black Bengal Goat. The buck produces semen and the semen volume is (0.5 ml/discharge), sperm Concentration (2.4 × 109/ml), mass motility (80 %), live spermatozoa (86.5%), and normal spermatozoa (89.3 %) (Mahal et al., 2013). It was stated that semen quality and attributes were influenced by body weight, body condition score, age, scrotal circumference, testicular circumference, breed, management, climatic, nutrition, technique for semen assortment, and level of sexual excitement (Furstoss et al., 2009; Akpa et al., 2013; Kumar et al., 2014; Agossou and Koluman, 2018; Gofur et al., 2019).

**Table 2.1: Characteristics of BBG semen**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Investigator | Semen volume (ml/ejaculate) | Sperm motility (%) | Sperm conc.  (× 106/ml) | Live sperm (%) | Normal (%) |
| Afrin et al. 2014 | 0.4–0.5 | 68.8–80.8 | 2110–2620 | 78.6–87.8 | 85.5–90.6 |
| Apu et al., 2008 | 0.58 ± 0. 03 | 77.8 ± 0.6 | 2797.2  ± 18.7 | - | - |
| Siddiqua et al., 2016 | 0.5 ± 0.3 | 80.8 ± 3.5 | - | 89.64 ± 5.1 | 90.84 ± 3.97 |
| Sultana et al. 2012 | 0.6–1.1 | 77.1–81.5 | 2827–3132 | 87.6–93.0 | 78.8–91.9 |

## 2.3. Evaluations of semen

Evaluation or analysis of semen is the most important and essential parameter offering high accuracy and reliability of semen to produce an Al dose. Spermatogenesis and fertility tests in males are 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 have been several 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 laboratories in developing countries (Pacey, 2010). Alternatively, different types of microscopes are the aids that help to investigate the spermatozoa. Light and phase contrast microscope which is commonly used to investigate the spermatozoa for their evaluation. The parameters that include routine semen evaluation are volume, density, sperm 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 its complex structure (Januskauskas and Zilinskas, 2002). Recently more attention has been given to evaluating semen by 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 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 it has many advantages of this technique, it is somehow quite impossible to apply everywhere because of its expensive and operation limits. compensate these hazards, 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), acrosomal 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).

## 2.4. Spermatozoa of Goat

The mammalian spermatozoa have a flattened head with an oval-shaped structure (Giarner, 2006). The size and shape of spermatozoa vary 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 other animal sperm. Head and acrosome are smaller in goats than other animal’s spermatozoa but the mid piece and the tail length of goat sperm is longer than other farm animal sperm. The average head length of goat spermatozoa is 10.04 µm; the width is 5.22 µm; the circumference is 27.18 µm, and the area is 38.69 µm. The acrosome area of sperm is 23.07 µm, and mid-piece and tail lengths are 17.03 µm and 77.97 µm, respectively. The mean overall length of goat spermatozoa is about 88.0 µm (Andraszek et al., 2014).

## 2.5. Color and consistency of semen

The quality of semen is determined by the number of sperms present per milliliter of semen. Semen can vary in color from white to milky or creamy white. A yellow color indicates a higher presence of riboflavin, while a brownish color is indicative of mostly dead sperm. The consistency of semen can be thick or thin, with thick consistency indicating a high concentration of spermatozoa (Saraswat et al., 2012). According to a study by Hahn et al. in 2019, among 20 collected semen samples, 80% had a yellow color and 20% had an ivory color. In terms of consistency, 70% of the samples were milky and 30% were creamy.

## 2.6. Volume of semen

Semen volume is an essential factor in the evaluation and preservation of semen in males (Ax et al., 2000). The volume of semen may vary depending on the breed, age, season of semen collection, level of nutrition provided to the buck, testicular size, and collection interval. The normal volume of semen for bucks is 0.5-1.5 ml (Hahn et al, 2019), while for 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). The overall mean value of Black Bengal buck semen ranges from 0.58 ± 0.17 ml to 1.04 ± 0.11 ml (Sultana et al., 2013; Apu et al., 2008). Sirohi breeding buck produces 1.06-0.01 ml of semen in the breeding season (Khadse et al., 2019), and Jamunapari bucks produce an average volume of 0.9 ± 0.2 ml (Hassan et al., 2010). While there is more variation in the volume of buck semen, limited research has been done to evaluate the volume of Jamunapari buck semen found in Bangladesh.

## 2.7. Concentration of spermatozoa

The concentration of spermatozoa in semen depends on various factors, such as age, breed, frequency of collection, number of ejaculations, and season of the year. In Black Bengal Bucks, Faruque et al., (2007) found an average sperm concentration of 2828 ±11.8 million per ml. However, Goswami et al. (2020) found that Beetal bucks and Sirohi bucks had varying sperm concentrations, with an average of 3356.73±83.06 million per ml and 3176.94±53.92 million per ml, respectively. Breed variation plays a significant role in determining sperm concentration. In goat semen, Singh et al., (1985) obtained a sperm concentration of 2619.58 to 2910.33x106/ml, while Khan (1999) found a concentration of 3777.93x106/m1. The collection frequency of ejaculation also affects sperm concentration. Thwaites (1995) found that four to eight daily ejaculations significantly lowered semen volume, sperm motility, sperm concentration, and the number of spermatozoa per ejaculation in rams. Conversely, Sharma et al., (1969) found that collecting semen once daily resulted in a higher concentration of sperm.

## 2.8. Motility of spermatozoa

Sperm motility is an important factor in determining the quality and viability of semen. It is the most common parameter used in semen evaluation to assess the quality of spermatozoa (Fonseca et al., 2005). Motile spermatozoa are indicative of mature sperm and rapid progressive motility is particularly important for fertility since these sperm can easily navigate the cervical mucus (Bjorndahl, 2010). Yodmingkwan et al., (2016) found that the motility of Boer buck fresh semen was 80.83 ± 3.06% and that it decreased in frozen semen when extended with different extenders. Similarly, Sultana et al., (2013) found that fresh semen motility of Black Bengal ranged from 77.07 ± 1.06% to 81.47 ± 1.84%, while frozen semen motility ranged from 48.15 ± 1.99% to 55.88 ± 2.97%.

## 2.9. Viability of spermatozoa

It is important to assess the viability of sperm before AI procedures. Preservation can damage the sperm membrane, causing it to lose permeability and die (Croass and Hanks, 1991). For successful conception, fresh sperm should contain more than 70% viable spermatozoa (Nilani et al., 2012). The viability of fresh and preserved semen depends on various factors such as the age, breed, diet, and semen volume extender of domesticated animals (Yodmingkwan et al., 2016; Kadirvel et al., 2009). In Bangladesh, Hassan et al. (2010) found that the viable sperm of Jamunapri goats was 90.3 ± 2.2%. Meanwhile, Hossain (2007) observed that the mean proportion of dead spermatozoa in Black Bengal buck fresh semen was 14.4±0.38% to 15.01±0.52%. After preservation with different semen volume extenders, the viability of goat spermatozoa varied significantly (Mara et al., 2007).

## 2.10. Functional integrity of spermatozoa (HOS test)

Integration of HOST into the sperm selection method may provide a beneficial tool for the selection of functional sperm. Recently, the hypo-osmotic swelling test has been proposed to potentially select sperm with intact membranes. 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 an increasing storage period. Vera-Munoz et al., (2011) found 68.1% and 48.8% HOST-positive sperm in fresh and post-thawed semen in bulls which affects fertility. So, integration of HOST into the sperm selection procedure may provide a valuable tool for the selection of functional sperm required for fertilization.

## 2.11. Morphology of spermatozoon

Morphology of spermatozoa means size, shape, and structure. Morphologically normal spermatozoa are important in determining fertility. There are different types of abnormality of spermatozoa recorded: macrocephalic, microcephalic, pear-shaped head, narrow head, stunted tail, bent tail, and dag defect (Koonjaenak et al., 2007). Normal and mature spermatozoa should have a neck and mid-piece short and no cytoplasmic droplets while immature spermatozoa consist of cytoplasmic droplets attached to the mid-piece area or tail (Knobil and Neill, 1998). However different factors affect sperm morphology. Different types of extenders and 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), reported 91.27±0.47 to 92.08±0.39% of normal sperm and the difference in buck to buck was non-significant. Sultana et al., (2013) found in Black Bengal buck 87.1±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).

## 2.12. Semen preservation

Sperm cryopreservation is an assisted reproduction technique (ART) for artificial insemination which allows us to use the semen from valuable sires and 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 to obtain satisfactory results. The success of artificial insemination (AI) is based on the ability to efficiently collect and perform cryopreservation of semen from superior quality bucks to use in does following generations (Amoah and Gelaye, 1990). There are 3 existing methods of semen preservation (fresh, refrigerated, and frozen) commonly used in goats worldwide (Leboeuf et al., 2000). Smith and Polge, (1950) worked as the pioneering goat semen cryopreservation. Barker, (1957) first proposed that the fertility of frozen-thawed goat sperm was too low to be useful different extenders and freeze techniques had been described in various animal species since then, particularly in bulls (Martin et al., 2004), goats (Jimenez-Rabadan et al., 2013 and Sharma et al., 2020) and ram (Munyai, 2012) to minimize detrimental effects of cryopreservation on spermatozoa 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 some common steps such as collection and extension of semen, the addition of cryoprotectant, cooling above 0°C, cooling below 0°C, storage, and thawing (Curry, 2007).

## 2.13. Semen collection and processing

There are some 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). The most preferred semen collection technique in Bucks are AV method. Artificial vagina (AV) is a technique which was first introduced by the Russian scientist. The AV method 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 (°C) above normal body temperature (Matshaba, 2010). The warm temperature from the water on the AV provides thermal and mechanical stimulation over the glans penis for the buck to ejaculate (Matshaba, 2010 and Munyai, 2012). The artificial vagina is lubricated for easy insertion of the penis. A doe (dummy) is restrained in a neck clamp and the buck is allowed to mount on her. Immediately when the buck mounts the doe, the penis is directed into the AV for ejaculation to take place in the collection glass.

## 2.14. Semen extenders

The main purposes of semen extender are to supply the sperm cells with a source of energy, protect the cells from cold shock, and maintain a suitable 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 suloxide DMSO), a buffer (Tris), one or more sugars (glucose, lactose, raffinose, saccharose, dextran or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin) (Sharma, 2018). 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 (Mollincau 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; Zanganch et al, 2013) have also been used with good results.

## 2.15. Role of egg-yolk

Egg yolk is a vital element 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 liquid storage and cryopreservation of most mammalian species, including bulls, rams, and bucks (Moussa et al., 2002). Egg yolk is a non-penetrating cryo-protective component and is globally used for semen preservation. Chicken egg yolk consists of phospholipids, cholesterol, and low-density lipoprotein. These are protective components that protect the membrane phospholipids 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 the extenders and also on the processes applied for the preservation of semen. A wide range of egg yolk concentrations has 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.

## 2.16. Short-time preservation or chilled semen

Semen preservation is the prime requirement for artificial insemination (AI) in Animals (goats, sheep, buffaloes, cows, horses, etc.). Chilled semen could be a valuable alternative to frozen semen with some beneficial points of view. It could maintain higher percentages of motile sperm in an AI dose compared with frozen-thawed semen at the same concentration. There are no hazardous effects of liquid nitrogen management; semen could be used even if they are bad freezers (Vera-Munoz et al., 2011). It is well known that the pregnancy rate is combatively higher in liquid semen than in frozen buck semen (Mara et al, 2007, Kharche et al., 2013) and easy for preservative.

## 2.17. Cryopreservation/freezing in Liquid N2

Cryopreservation is an updated procedure to allow specific benefits to the livestock industry (Bucak et al., 2009). During this process, semen is exposed to cold shock and atmospheric oxygen which helps to increase the susceptibility to lipid peroxidation (Bucak et al., 2008). Cryopreservation of a mammalian sperm is a complicated technique that requires careful consideration of a number of variables in order to get satisfactory results. To ensure 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 of 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 bulb-urethral 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 state techniques have been studied to reduce these effects on 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 Al compared to other storage semen because frozen semen can be stored for an unlimited period and easily transported to any area.

## 2.18. Vitamin C (L-Ascorbic acid)

Ascorbic acid is a water-soluble vitamin that has been shown to have antioxidant properties. L-ascorbic acid commonly named vitamin C. Goats' semen is highly sensitive to lipid peroxidation that occurs as the result of the oxidation of membrane lipid through superoxide, hydrogen peroxide, and hydroxyl radicals. The structures of the lipid matrix are destroyed by the spontaneous lipid peroxidation of the sperm membrane. The actions of the reactive oxygen species lead to the impairments of sperm functions like as sperm motility, functional membrane integrity, leakage of intracellular enzymes, and damage of the DNA through oxidative stress (Alvarez and Storey, 1989). Naturally, ascorbic acid is found in the epididymal fluid of the semen (Azawi et al., 2013). When the amounts of the semen are stored in vivo, natural antioxidants do not counter the ROS (Reactive Oxygen Species) production and lipid peroxidation. Intramuscular (Sonmez and Demici, 2003) and subcutaneous (Fazeli et al., 2010) vitamin C administration has increased the semen quality, and effects were evident up to 30 days after the cessation of injections. Supplementation of this antioxidant (ascorbic acid) in semen diluents improves the motility during chilled semen storage at 5°C (Ball et al. 2001) and ascorbic acid prevents the damage induced by free radicals during freezing and therefore improves the fertility of liquid ram spermatozoa. It neutralizes hydroxyl, superoxide, and hydrogen peroxide (H2O2) radicals and protects from sperm agglutination. Hussein (2013) mentioned that the supplementation of vitamin C in preservation media could improve the longevity and quality of chilled and ram semen. The beneficial effect of supplementation vitamin C reduced ROS generation in diluted ram semen was in accordance with the results obtained by Asghari (1999). Saraswat et al., (2012) observed that supplementation of ascorbic acid (9mM) is capable of protecting sperm from reactive oxygen species, lipid peroxidation, and DNA-damage. The higher amounts of vitamin C (above 2.5mM) addition were proved harmful to sperm motility in frozen-thawed bull semen (Beconi et al., 1993). The reduction in motility might be due to the acidic nature (pH 2) of the vitamin C which reduces the pH of the diluents.

## 2.19. Glutathione (GH)

Glutathione is a molecule that is present in several mammalian cells at mM level. It reacts directly with ROS, and it is a cofactor for glutathione peroxidase (GSHPx) that helps in catalyzing the reduction of toxic H2O2 and other hydroperoxides. It protects against oxidative stress (Bucak et al., 2008). Glutathione works as an enzymatic antioxidant that reduces hydrogen peroxides to water and alcohol. It suppresses the formation of free radicals by decomposing them, protecting sperm cells from continuous oxygen (O2) toxicity and lipid peroxidation. It also plays a crucial role in removing O2 produced by NADPH oxidization in neutrophils, reducing LPO, and preventing oxidative damage. During semen preservation, oxidative damage occurs due to the production of reactive oxygen species by the cellular components of semen. This production of ROS is possibly one of the main causes of the decline in motility and fertility during storage due to lipid peroxidation. The effects of lipid peroxidation in sperm cells include irreversible loss in motility, viability, sperm abnormality, damage to the sperm DNA, and fertility (De Lamirande and Gagnon, 1992; Aitken, 1994; Maxwell and Watson, 1996). Frozen-thawed ram/buck semen shows serious cryopreservation damage and highly decreased fertilizing capacity. The semen antioxidant system of different animals is so weak that sperm easily undergo lipid peroxidation (Foote et al., 2002). So, supplementing glutathione (GSH) in chilled and frozen semen can improve sperm motility and preservation time (Gupta and Tripathi, 1984; Slaweta and Laskowska, 1987).

# CHAPTER 3: MATERIALS AND METHODS

## 3.1. Description of the study area

The study was conducted in Chattogram metropolitan area, Bangladesh. It is the second-largest economic city in Bangladesh and is located at 22°20'18.24" N and 9149'54.05" E. It has tropical monsoon climatic conditions which are characterized by an annual average temperature of 13 to 32°, a humidity of 70-80%, and rainfall of 5.6 mm to 725.0 mm (Khan et al., 2019). In this Chottogram metropolitan city, the goat population is about 30,320 (DLS, 2017). The study was conducted from December, 2021 to June, 2022 during this period semen sample were collected from bucks to study the different parameters for achieving study objectives. This study was conducted in the Theriogenology laboratory, Department of Medicine and Surgery of Chattogram Veterinary and Animal Sciences University (CVASU),

## 3.2. Animal selection and management

Five goats were selected based on their physical features: two Jamunapari, two Black Bengal bucks, and one doe. These experimental goats were purchased from the local market and were previously used in the Banbeis project (budget code 4829.1). All the animals used during the study were kept in a research goat shed located near the Theriogenology Laboratory of the Department of Medicine and Surgery at the Faculty of Veterinary Medicine, CVASU, in Chattogram, Bangladesh. The bucks were fed twice a day with a diet containing 450g of commercial concentrate in mash form (with a crude protein content of 120g/kg DM and energy content of 10.4 MJ ME/kg DM), 350g of grain, and 1.5kg of green grass in the morning, and 500g of commercial concentrate and 1.5kg of green grass per head in the afternoon. The does were raised in a semi-intensive system and fed by grazing on pasture with a small amount of concentrate (300g in the morning and 400g in the evening per animal). Additionally, all animals had ad-libitum access to clean and safe water. Throughout the study period, all the animals were regularly dewormed with different anthelmintics (Fenazol®, LT-vet®, and Amectin plus® from ACME Laboratories Ltd. Bangladesh), and vaccinated with Peste des petit ruminants (PPR) (PPR® Vaccine, LRI, Bangladesh) according to a predetermined schedule. Regular clinical and physical examinations were conducted to ensure the health of the animals, and treatment was given as necessary.



Figure 3.1: Animal shed and Jumunapari buck-1

Figure 3.2: Dumy doe Figure 3.3: Jamunapari buck-2

## 3.3. Preparation of semen extenders:

In the study, three different types of semen extenders were used. These were 1. TCEY-1: Tris citrate egg yolk (TCEY) 5% based extender (Control). 2. TCEY-2: TCEY added with three different concentrations of Glutathione (3mM GH, 5mM GH, 7mM GH) from Sisco Research Laboratories Pvt. Ltd., Moharastroh, India. 3. TCEY-3: TCEY added with three different concentrations (3mg vit-C, 5mg vit-C and 7mg vit-C) of Vitamin-C from Research-lab fine chem. Industries, Mumbai, India. To prepare a stock solution, 3.63g of Tris (GPR®, BDH Laboratory Ltd., and England), 2.0g of citric acid (Emprove®, Merck Ltd., and Germany), and 0.5g of fructose (D-Fructose, Merck Ltd., India) were dissolved in deionized water to make a 100 ml solution. The solution was then stored at 4°C for a maximum of two weeks (Rekha et al., 2016). On the semen collection day, 5% (w/v) egg yolk and combine 10000 IU of penicillin, and 100mg streptomycin (Streptopen®,Ranata Pharmaceutical Ltd. Bangladesh) were added to the stock solution according to the study design. After that, the total volume of the extender was divided into three separate beakers and marked as i) TCEY-1(control) ii) TCEY-2 (Glutathione) and iii) TCEY-3(Vit-C). The TCEY-2 and TCEY-3 parts of extender again divided and taken into three for each different graduated collection tube separately and then name as 3mM GH, 5mM GH, 7mM GH for Glutathione extender as well as 3mg vit-C, 5mg vit-C and 7mg vit-C for Vitamin-C extender. Accordingly, the tube containing the extender was added with a marked amount of Glutathione and Vitamin-C, respectively. The final semen extender containing a beaker and graduated collection tube was kept at 35ºC temperature until mixed with the semen for chilling.

For freezing the semen, extenders were prepared following the same procedure as the extender for semen chilling except additional 7% glycerol. In brief, the stock solution of the extender for freezing the semen was divided into two parts and named Part A without glycerol and Part B with glycerol. The glycerolized parts of the semen extender Part B were kept in the refrigerator and Part A non-glycerolized parts of the semen extender were kept at 33-35ºC temperature in a water bath until use.





Figure 3.4: Preparing semen extender and semen mixing

## 3.4. Semen collection

Before the collection of semen, all the bucks were trained to use a specific artificial vagina designed for sheep and goats (Fa. Minitube, Tiefenbach). All glassware for collection and handling was cleaned and sterilized using high-pressure steam followed by drying and warming at 35°C. The semen was collected from bucks once per week by the Artificial Vagina (AV) method. Water at 51-52°C was filled in the rubber tube for stimulation of the penis with the vaginal temperature (Hahn et al, 2019). A total of 18 ejaculates were collected from bucks during the monsoon season (December 2021-June 2022). The collection was always performed in the morning (6:00-8:00 AM).



Figure 3.5: Collection of semen from Jamunapari buck-1

## 3.5. Evaluation of semen

After collection, semen was kept at 33-35°C in a water bath until the fresh semen evaluation and extenders were added to it. Macroscopic semen evaluation was done by the naked eye such as semen color, and volume. Semen volume was directly measured using the graduated collecting tube and pH was measured by pH paper (McolorpHast™, Merck, Germany). The color was estimated by visual inspection and density was scored by making the tube slant with a range 1-5 score. The scoring system for semen density was as follows: 1- watery (400-1000 x 10⁶sperm/ml); 2- thin milky (1000-2500 x 10⁶ Sperm/ml); 3- thin creamy (2500-3500 x 10⁶ sperm/ml); 4- creamy (3500-4500x l0⁶ sperm/m); and 5- thick creamy consistency (4000-6000x 10⁶ 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). A drop of 20µl of semen was placed on a pre-warmed (37°C) slide without a cover slip and examined under a phase-contrast microscope 100x to assess mass activity as wave motion. Mass motility was estimated by assessment of wave motion of fresh undiluted semen at 100x magnification (0–5 scale). 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, 5µl of diluted semen was placed on a pre-warmed (57°C) slide, covered with a cover slip, and evaluated (400x). The concentration of spermatozoa was determined using a Neubauer counting chamber of a hemocytometer (Marienild, Germany), and the total number of spermatozoa was calculated by multiplying the volume of the ejaculate by the concentration.

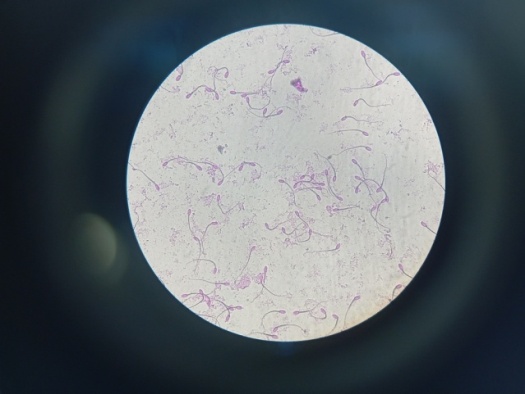
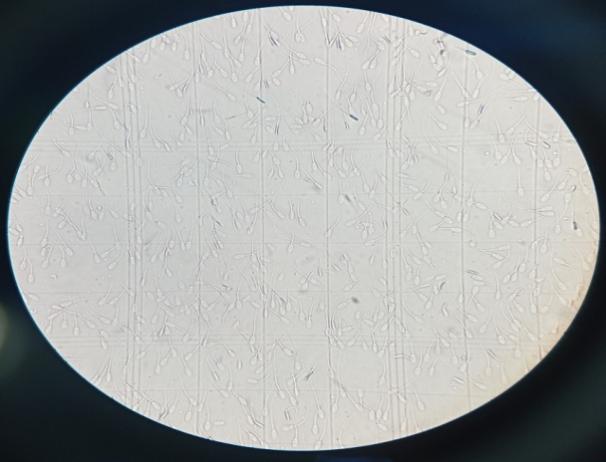
An eosin-nigrosin stain was used to determine the viability of spermatozoa. An equal amount of semen and one drop of eosin-nigrosin stain were placed on a clean slide and mixed with a clean stick. Thin smear was dried in the air, and examined under (40x). At least 200 spermatozoa were examined from each smear to calculate the percentage of live spermatozoa.

Figure 3.5: Semen evaluation and observation. a. mass motility test. b. Sperm abnormality count c. crooked tail abnormality d. sperm count

**a b**

**c d**

a b

c d

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 considered as live (Mortimer, 1994). A hypo-osmotic swelling test (HOST) was used to detect functionally integrated spermatozoa. The HOS (Hypo-Osmotic Solution) solution was prepared by mixing 9g of fructose and 4.9 g of sodium citrate into 1L of deionized water, 20:1 of sperm were mixed with 200µl of HOS solution and incubated for 60 minutes at 37°C. After incubation, 5µl of HOS mixed semen was placed onto a pre-warmed (37°C) side, covered with a cover slip, and examined under a microscope (400x). 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).

## 3.6. Semen preservation

After collection of buck semen was preserved by using diluents as the form of chilled and frozen. Initially ejaculated semen was examined macroscopically and microscopically than mass motility and concentration (10⁶/ml) of spermatozoa were evaluated. All ejaculates were stored in falcon tube 35°C separately for Jamunapari and Black Bengal buck semen sample individually after primary evaluation. Then the semen was divided into different parts of different diluents of different concentration for chilling and frozen semen of Jamunapari buck semen and each part of semen was taken on falcon tube respectively.

## 3.6.1 Preservation of semen in chilling temperature

After preliminary evaluation of fresh semen, the required volume of extender was calculated, then diluted those fractions to previously made; 5% TCEY-1 (control), TCEY-2 (3mM GH, 5mM GH, 7mM GH) and TCEY-2 (3mg/ml vit- C, 5mg/ml vit- C, 7mg/ml vit- C) and kept at 4-5°C until evaluation on refrigerator. The chilled semen was held at the temperature of 4-5 minutes before evaluation to adjust the environmental temperature. On Days 2nd, 4th, 6th the chilled semen was examined for motility, viability, functional integrity (HOS test), and normal morphology.

## 3.6.2 Cryopreservation of semen

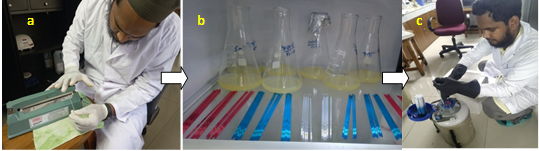
For freezing, the required semen extender volume was calculated and then fresh semen samples were diluted with the different extender parts without glycerol; TCEY-1 (control), TCEY-2 (3mM GH, 5mM GH, 7mM GH) and TCEY-3 (3mg/ml vit-C, 5mg/ml vit-C, 7mg/ml vit-C) separately at room temperature and kept at 4°C for the next 2 hrs for equilibrium. Then extender parts with glycerol such as TCEY-1 (control), TCEY-2 (3mM GH, 5mM GH, 7mM GH and TCEY-3 (3mg/ml vit-C, 5mg/ml vit-C, 7mg/ml vit-C) were added, respectively. After that, the final mixed extended semen was loaded into a 0.25 ml mini semen straw (Minitube (GmBH, Germany), sealed by the sealer machine and held for the next 2 hrs at 4°C. All straws were exposed to liquid nitrogen vapor for 6 minutes after 4 hours of equilibration before being transferred to liquid nitrogen (-196°C) for storage until thawing. The final concentration of frozen semen was 100 million/ml. Frozen semen straw was evaluated for individual motility, viability, functional integrity and normal-abnormal morphology on Days 3rd, 10th, and 20th after cryopreservation.

Figure 3.6: Semen straw sealing (a), storage (b) and preservation in liquid N2 tank (c)

## 3.7 Statistical analysis

All collected data were tabulated in MS Excel first and then analyzed by using STATA software (version 13.0 for Windows, Stata Corp. College Station, USA). For statistical analysis, an independent samples t-test was performed to compare semen parameters between fresh semen, and a one-way ANOVA procedure was used to evaluate the treatment effect on semen parameters. The collected data were expressed as mean ± SEM. The significance of differences between means was tested using Tukey’s honestly significant difference test (Tukey’s HSD). Statistical significance was considered when P ≤ 0.05.

# CHAPTER 4: RESULTS

## 4.1. Comparative evaluation of fresh semen quality between Jamunapari and Black Bengal goats

In fresh semen quality, the Jamunapari buck had a higher semen volume (P˂0.05) than in Black Bengal buck. However, the density and concentration of semen were significantly increased (P˂0.05) in Black Bengal buck semen than in Jamunapari buck. Mass motility, viability, and functional integrity of semen did not differ in significant level between these two experimental breeds. The semen color and PH were not significantly differs in these two breeds of buck (Table 4.1).

**Table 4.1: Comparative evaluation of fresh semen in Jumunapari and Black Bengal goats**

|  |  |  |  |
| --- | --- | --- | --- |
| **Semen**  **Parameters** | **Bucks** | | |
| **Jumunapari**  **n=18** | **Black Bengal**  **n=18** | **P value** |
| Volume(ml) | 1.05± 0.38a | 0.51±.12b | <0.001 |
| PH | 6.66±0.16a | 6.36±.15a | 0.06 |
| Color | Creamy | Creamy |  |
| Density (0-5 score) | 4.13 ± 0.30a | 4.37 ± 0.22b | 0.03 |
| Mass motility  (0-5 score) | 4.73 ± 0.45a | 4.41 ± 0.33a | 0.02 |
| Concentration (x109 /ml) | 3.50± 0.14a | 3.71±0.16b | 0.26 |
| Viability (Live)% | 91.53± 4.5a | 91.84±4.8a | 0.39 |
| Functional integrity  (HOS test) | 85.19± 4.08a | 85.08±4.20a | 0.95 |

[Here, values are mean±SEM. a-b means bearing different superscript are significantly different in row at P≤0.05]

## 4.2. Effects of antioxidants on the quality of chilled semen in Jumunapari buck

## 4.2.1. Motility

Table 4.2 represented the motility of chilled semen in Jamunapari buck. The results revealed that the antioxidant either Glutathione or Vit-C improved the semen quality than the control group (p≤0.05) during chilling. Between antioxidant the Glutathione showed the high quality of Jamunapari buck semen than Vit C when chilling (p≤0.05). The studied conducted by adding different concentration of Glutathione and Vit-C separately in TCEY extender. The concentrations were 3mM, 5mM and 7mM for Glutathione and 3mg, 5 mg and 7 mg for Vit-C, respectively. It was showed that 5mM Glutathione (86.83±1.56) added TCEY extender maintained the highest quality of motility in chilled semen followed by 3mM GH (85.06±1.44) and 7mM GH (80.43±1.81). On the other hand, Vit-C added TCEY extended chilled semen proved that 7mg vit-C (84.17±1.19) maintained highest quality compared to 3mg vit-C (81.33±1.36) and 5mg vit-C (81.04±0.33), respectively (Table 4.2). The table was also represented the effect of chilling time on quality of Jamunapari buck semen. It was observed that preservation time degraded the motility of semen when chilling in all three groups (TCEY-1, TCEY-2 and TCEY-3). The sperm motility was significantly degraded from day 2 to day 4 and day 6 (p≤0.05) in all groups of semen.

**Table 4.2: Effects of antioxidants on motility of Jumunapari buck chilled semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Motility (%)** | | |
| **Preservation time (Day)** | | |
| **2** | **4** | **6** |
| **TCEY-1** | **Control** | 78.88±1.73ax | 73.28±1.34ax | 57.08±2.36az |
| **TCEY-2** | **3mM GH** | 85.06±1.44bx | 75.00±1.42ay | 59.04±2.49az |
| **5mM GH** | 86.83±1.56bx | 79.53±1.61by | 62.14±2.08az |
| **7mM GH** | 80.43±1.81ax | 75.28±1.22ax | 59.21±2.85az |
| **TCEY-3** | **3mgVit-C** | 81.04±0.33ax | 74.90±1.77ax | 57.33±1.33az |
| **5mgVit-C** | 81.33±1.36ax | 75.53±1.26ax | 57.62±2.79az |
| **7mgVit-C** | 84.17±1.19bx | 75.59±1.42ay | 58.56±2.98az |

[a- b means bearing different superscripts in a column differ significantly between treatments at P≤0.05. x-z means bearing different superscripts in a row differ significantly between treatments at P≤0.05, TCEY= Tris citrate egg yolk, vit-C= Vitamin C(L-ascorbic acid), GH=Glutathione]

## 4.2.2. Viability of sperm

The effect of antioxidant on viability of chilled semen was presented in Table 4.3. The results showed the TCEY added GH and vit-C semen extender worked positively to maintain higher percentages of viable sperm compared to TCEY-1(control) (p≤0.05) in three different chilling times. Within these two antioxidants, TCEY-2 (added GH) extender showed highest percentages of viable sperm than TCEY-3 (added vit-C) semen extender (p≤0.05) in each day of preservation time. Among the different amount of GH in TCEY-2 extender, it was observed that 5mM GH (86.83±1.56) added extender produced highest number of viable sperm compared to 3mM GH (84.59±1.42) and 7mM GH (83.96±1.70) added extender. In case of Vitamin C, TCEY-3 extender added 7mg vit-C (85.91±1.38) revealed highest number of viable sperm followed by 5mg vit-C (84.37±2.10) and 3mg vit-C (83.81±1.79) added in TCEY-3 extender in each day of preservation time. The viability of spermatozoa was decreased with advancing the preservation time (p≤0.05) (Table 4.3).

**Table 4.3: Effects of antioxidants on sperm viability of Jumunapari buck chilled semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Viability (%)** | | |
| **Preservation time (Day)** | | |
| **2** | **4** | **6** |
| **TCEY-1** | **Control** | 81.87±1.47ax | 72.06±1.43ay | 54.81±2.59az |
| **TCEY-2** | **3mM GH** | 84.59±1.42ax | 75.88±1.83ay | 63.06±2.21bz |
| **5mM GH** | 86.83±1.56bx | 80.76±1.61bx | 67.20±2.14bz |
| **7mM GH** | 83.96±1.70ax | 76.02±1.02ay | 59.22±3.14az |
| **TCEY-3** | **3mgVit-C** | 83.81±1.79ax | 74.21±1.53ay | 60.50±1.25az |
| **5mgVit-C** | 84.37±2.10ax | 75.24±1.96ay | 61.05±2.79az |
| **7mgVit-C** | 85.91±1.38bx | 77.88±1.83by | 66.17±2.43bz |

[a- b means bearing different superscripts in a column differ significantly between treatments at P≤0.05. x-z means bearing different superscripts in a row differ significantly between treatments at P≤0.05, TCEY= Tris citrate egg yolk, vit-C= Vitamin C(L-ascorbic acid), GH=Glutathione]

## 4.2.3. Sperm morphology

The normal morphology of spermatozoa was studied in control and antioxidant (GH and vit-C) added semen extenders in three different preservation times including days 2, 4, and 6 in chilling temperatures. There was no significant effect of studied semen extenders (P>0.05) in normal sperm count in day2, 4, or 6. It was also shown that preservation time during chilling of semen has no effect on normal sperm count in different types of extenders (Table 4.4).

**Table 4.4: Effects of antioxidants on normal sperm count of Jumunapari buck chilled semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Normal sperm (%)** | | |
| **Preservation time (Day)** | | |
| **2** | **4** | **6** |
| **TCEY-1** | **Control** | 96.00±1.38 | 95.63±1.38 | 95.33±1.45 |
| **TCEY-2** | **3mM GH** | 96.33±1.68 | 95. 28±2.03 | 95.51±2.23 |
| **5mM GH** | 96.93±2.40 | 95.69±2.83 | 96.48±1.73 |
| **7mM GH** | 96.41±2.02 | 96.75±1.67 | 95.83±2.26 |
| **TCEY-3** | **3mgVit-C** | 95.73±1.65 | 95.40±2.85 | 96.78±1.44 |
| **5mgVit-C** | 95.12±2.13 | 95.69±1.70 | 95.48±2.10 |
| **7mgVit-C** | 96.09±1.91 | 94.92±2.37 | 96.48±0.48 |

[TCEY= Tris citrate egg yolk, vit-C= Vitamin C(L-ascorbic acid), GH=Glutathione]

## 4.2.4 Functional integrity of sperm

The results showed antioxidants positively influenced the functional integrity of sperm. TCEY-2 extender 5mM GH (74.22±1.72) remained the highest percentages of functional integrated sperm followed by 7gm vit C (72.35±1.63) added and control (63.18±2.76) types of extenders (P≤0.05) in each observation time during chilling. Similar to the above results increasing the chilling time influenced negatively the functional integration of sperm in all three types of semen extenders (P≤0.05).

**Table 4.5: Effects of antioxidants on functional integrity of Jumunapari buck chilled semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Functional integrity** | | |
| **Preservation time (Day)** | | |
| **2** | **4** | **6** |
| **TCEY-1** | **(Control)** | 63.18±2.76ax | 60.27±1.63ax | 41.00±2.10az |
| **TCEY-2** | **3mM GH** | 73.79±2.47bx | 62.08±2.15ay | 45.28±2.15az |
| **5mM GH** | 74.22±1.72bx | 68.48±1.52by | 50.95±1.68bz |
| **7mM GH** | 70.00±1.57bx | 68.13±2.06bx | 48.46±2.12bz |
| **TCEY-3** | **3mgVit-C** | 69.87±2.06bx | 61.25±1.50ay | 43.12±2.05az |
| **5mgVit-C** | 70.17±1.35bx | 63.83±1.76ay | 42.01±2.00az |
| **7mgVit-C** | 72.35±1.63bx | 69.71±1.92by | 42.36±1.69az |

[a- b means bearing different superscripts in a column differ significantly between treatments at P≤0.05. x-z means bearing different superscripts in a row differ significantly between treatments at P≤0.05, TCEY= Tris citrate egg yolk, vit-C= Vitamin C(L-ascorbic acid), GH=Glutathione]

# 4.3. Effects of antioxidants on the quality of frozen semen in Jamunapari buck

## 4.3.1. Motility

Antioxidants including Glutathione and Vitamin C in three different fractions: 3mM GH, 5mM GH, 7mM GH and 3mg vit-C, 5mg vit-C, and 7mg vit-C, respectively used to evaluate the effects of antioxidants on the quality of post-preserved Jamunapari buck semen in frozen method. Table 4.6 presents the effects of antioxidants on the motility of sperm in different preservation times (day 3, 10, and 20). Motility of sperm was increased significantly in all three concentrations of Glutathione added in TCEY extenders compared to Control (51.38±0.96) and Vit-C added extenders (P≤0.05). Among the different concentrations of TCEY-2 and TCEY-3, the results revealed that 5mM GH (52.33±0.14) and 7mg vit C (51.38±0.96) added semen extenders showed the highest number of motile sperms compared with rest two fractions of concentrations in each type of extender (P≤0.05). In TCEY-3 extenders, when concentrations were increased gradually the effects on post-thawed sperm motility also increased (P≤0.05) in each observation. In contrast, chilled semen increasing the preservation time did not affect the motility of sperm (Table 4.6).

**Table 4.6: Effects of antioxidants on motility of Jumunapari buck frozen semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Motility (%)** | | |
| **Preservation time (Day)** | | |
| **3** | **10** | **20** |
| **TCEY-1** | **(Control)** | 41.71±0.09a | 40.55±1.88a | 40.05±1.11a |
| **TCEY-2** | **3mM GH** | 48.03±1.55b | 47.24±1.42b | 46.13±0.71b |
| **5mM GH** | 52.33±0.14c | 52.25±3.98c | 51.18±2.56c |
| **7mM GH** | 46.92±1.60b | 46.55±2.03b | 46.05±1.25b |
| **TCEY-3** | **3mgVit-C** | 43.44±2.07a | 42.17±3.60a | 40.71±1.04a |
| **5mgVit-C** | 47.80±0.37b | 46.89±2.77b | 46.37±2.45b |
| **7mgVit-C** | 51.38±0.96c | 50.63±2.93c | 50.51±2.53c |

[Here, values are mean±SEM. a- b-c means bearing different superscripts in a column differ significantly between treatments at p≤0.05, TCEY= Tris citrate egg yolk, vit-C= Vitamin C (L-ascorbic acid), GH=Glutathione

## 4.3.2. Viability of sperm

Table 4.7 shows the results of Glutathione (TCEY-2) and vitamin-C (TCEY-3) added TCEY extenders' effect on sperm viability. The effect of Glutathione on sperm viability was significantly higher compared with control and Vitamin-C added semen extenders (P≤0.05). The viability percentages of all fractions of glutathione added TCEY treatments were significantly increased in three observations than control (42.41±3.06) and then vit-C added extender (P≤0.05). 5mM GH (48.91±4.88) and 7mg vit-C (50.88±3.870) added TCEY extenders were better at maintaining higher number of sperm viable than 3mM GH, 7mM GH and 3mg vit-C, 5mg vit-C, respectively in Glutathione and vit-C supplemented semen extenders (P≤0.05). Similar to the motility of frozen Jamunapari buck semen, increasing the preservation time was not affects the viability of sperm with studied extenders (Table 4.6).

**Table 4.7: Effects of antioxidants on sperm viability of Jumunapari buck frozen semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Viability (live %)** | | |
| **Preservation time (Day)** | | |
| **3** | **10** | **20** |
| **TCEY-1** | **(Control)** | 42.41±3.06a | 41.52±2.38a | 39.34±3.05a |
| **TCEY-2** | **3mM GH** | 48.91±4.88b | 48.52±3.97b | 47.15±3.73b |
| **5mM GH** | 54.85±4.5c | 54.06±4.47c | 53.35±2.51c |
| **7mM GH** | 52.00±7.84c | 51.12±4.81c | 51.52±1.32c |
| **TCEY-3** | **3mgVit-C** | 44.32±4.67b | 44.05±1.93a | 43.45±3.9b |
| **5mgVit-C** | 47.14±3.50b | 45.49±2.7b | 45.09±2.82b |
| **7mgVit-C** | 50.88±3.87c | 48.65±2.40b | 47.50±2.22b |

[Here, values are mean±SEM. a- b-c means bearing different superscripts in a column differ significantly between treatments at p≤0.05,TCEY= Tris citrate egg yolk, vit-C= Vitamin C(L-ascorbic acid), GH=Glutathione]

## 4.3.3. Sperm morphology

Table 4.8 shows the findings for normal sperm count rate extending Jamunapari buck semen with control, and antioxidant (GHS and vit C in three different fractions) on day 3, day 10, and day 20 post-preserved. 5mM GHS added with TCEY semen extenders maintained the normal sperm in higher percentages than control and other fractions of GHS and vit C treated semen extenders studied (P<0.05)on day10 and 20 of post-preserved observation(P<0.05). The advancing preservation time did not affected the normal percentages of sperm (Table 4.7)

**Table 4.8: Effects of antioxidants on normal sperm count of Jumunapari buck frozen semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **Normal sperm (%)** | | |
| **Preservation time (Day)** | | |
| **3** | **10** | **20** |
| **TCEY-1** | **(Control)** | 94.02±1.88 | 93.52±1.87a | 90.85±1.65a |
| **TCEY-2** | **3mM GH** | 96.42±42 | 94.31±2.3a | 92.51±2.23a |
| **5mM GH** | 98.98±1.54 | 97.33±1.84b | 97.05±1.25b |
| **7mM GH** | 95.24±2.4 | 94.85±4.26a | 93.85±2.26a |
| **TCEY-3** | **3mgVit-C** | 95.16±3.24 | 95.06±2.06a | 93.23±1.73a |
| **5mgVit-C** | 96.87±1.82 | 95.27±2.20a | 95.09±1.13a |
| **7mgVit-C** | 96.59±2.19 | 96.66±1.68a | 94.21±2.36a |

[Here, values are mean±SEM. a- b means bearing different superscripts in a column differ significantly between treatments at p≤0.05, TCEY= Tris citrate egg yolk, vit-C= Vitamin C (L-ascorbic acid), GH=Glutathione]

## 4.2.4. Functional integrity of sperm

The findings of the HOS test were performed on frozen semen to observe any effects on the functional integrity of Jamunapari buck semen on day 3, day 10, and day 20 of freezing (Table 4.9). Antioxidant-treated semen extenders influenced the functional integrity of sperm than the TCEY treated as control. Among all the fractions of antioxidant concentration for GH and vit C in TCEY extenders 5mM (42.09±3.35), 7mM GH, and 7mg vit-C (39.87±3.40) showed a significantly positive effect on functional integrated sperm than the control (34.11±0.09) and others group(P≤0.05) on each preservation time. It was also observed that time did not affect the functional integrity of sperm.

**Table 4.9: Effects of antioxidants on functional integrity of Jumunapari buck frozen semen in three different preservation times**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Extenders** | | **HOS test (%)** | | |
| **Preservation time (Day)** | | |
| **3** | **10** | **20** |
| **TCEY-1** | **(Control)** | 34.11±0.09a | 33.51±1.88a | 30.55±2.11a |
| **TCEY-2** | **3mM GH** | 36.97±3.12a | 35.97±3.27a | 34.81±3.01a |
| **5mM GH** | 42.09±3.35b | 42.78±2.80b | 40.78±3.14b |
| **7mM GH** | 40.98±2.84b | 39.24±2.54b | 39.08±1.34b |
| **TCEY-3** | **3mgVit-C** | 31.16±1.52a | 30.89±3.01a | 29.84±3.33a |
| **5mgVit-C** | 34.88±2.21a | 34.63±0.90a | 32.27±2.03a |
| **7mgVit-C** | 39.87±3.40b | 36.23±2.29a | 36.66±1.18b |

[Here, values are mean±SEM. a- b means bearing different superscripts in a column differ significantly between treatments at p≤0.05, TCEY= Tris citrate egg yolk, vit-C= Vitamin C(L-ascorbic acid), GH=Glutathione]

# CHAPTER 5: DISCUSSION

## 5.1. Fresh semen evaluation of goat

In this study, the goat of both Jamunapari (JP) and Black Bengal (BB) semen were evaluated with their semen in chilled and frozen form with different antioxidant mixed extenders. In fresh semen evaluation, the semen volumes of the JP breed were found almost double of the BB goat. The overall semen volume of the experimented goats was around 1.05 in JP and 0.5 ml in BB buck per ejaculation which could be considered a normal volume of the goat as a similar volume was reported by Hahn et al. (2019); Sultana et al. (2013), Apu et al. (2008); Hassan et al. (2010).

Mass motility and sperm concentration of semen were slightly more in BB than in JP. The sperm concentration of JP was found 3.50± 0.14x109 per ml of semen which is more than the concentration reported by Reza, (2021) (3.17±0.2x109). The sperm concentration Of BB 3.71±0.16x109 was also found higher than Faruque et al. (2007) who reported 2.828 ± 0.11x109 /ml sperm. The semen density was found higher in BB compared to the JP is also supported by Reza, (2021)

Other parameters like semen color, pH, live sperm percentage, and functional integrity differences were insignificant between the breeds. However, the live sperm percentage 91.53±4.5 in JP to 91.84±4.8 in BB buck semen is higher than the viability reported by Hossain (2007); Kharche et al. (2013); Reza, (2021). Functional integrity (HOST+ve) of spermatozoa of fresh semen 85.19±4.8 in JP and 85.08±4.20 in BB buck semen in this study was similar to Gojen et al. (2016) who found 85.37±0.85% in Black Bengal semen but higher than the findings of Deori et al. (2018) who reported 66.95 ± 0.74% functional integrity. Berry et al., 2019 reported integrity differences among the goat breeds which was insignificant in this study. The pH of the study was found 6.66±0.16 (JP) to 6.36±0.15 (BB) which is similar to Hahn et al. (2019) who reported 6.4-7.0 pH of goat semen and below 6.5, the motility and metabolism of the sperm are gradually reduced.

## 5.2. Effect of Glutathione as an antioxidant in preserved goat semen

Sperm motility is a crucial factor for reproductive efficiency as it greatly determined the semen quality for cryopreservation and fertilization. In this study, individual motility was affected by the semen extender where glutathione (GH) seem to improve sperm motility significantly compared to the control treatment especially with 3mM and 5mM GH in chilled semen (day2 and 4) as well as in frozen semen by all concentrations on day3,10 and 20. In the past, GH supplementation had been shown to improve sperm motility in goats (Sinha et al., 1996, Zou et al., 2021) as well as other species like the bull (Ansari et al., 2012; Gangwar et al., 2018), boar (Yeste et al., 2014), dog (Ogata et al., 2015) and rabbit (Ahmad et al., 2021), mostly because GH causes a decrease in ROS. According to Stradaioli et al. (2007), the bovine semen extender has GH levels that have greater sperm motility and integrity compared to egg yolk tris-citrate extenders and can reduce oxidative damage to spermatozoa that have survived freezing and thawing methods. However, Ogata et al. (2022) reported no significant effect of GH on sperm motility in bulls which was supported by similar reports of Tuncer et al., (2010) and Perumal et al., (2011).In this study, 5mM GH showed the highest significance sperm motility followed by 3mM and 7mM GH concentration respectively on both chilled and frozen semen. This concentration however contradicted Zou et al., (2021) result, which demonstrated that when the glutathione content in the diluted solution was 2mmol/L, the motility performance of the goat sperm after thawing was greatly increased, but greater glutathione concentrations had a detrimental effect on sperm motility after thawing.

The viability of sperm was improved significantly by the 3mM and 5mM GH semen extender in chilled semen for days 2, 4, and 6. The viability of sperm was also improved in frozen semen on days3, 10, and 20 with all GH addition compare to the control especially with 5mM and 7mM concentration. In sperm morphology, the normal sperm percentage was not influenced by the GH addition on days 2, 4 and 6 in chilled semen. A similar result was also found in frozen semen on day 3. However, the normal sperm percentage was increased in the GH extender on day10 and 20 with 5mM concentration. Sperm functional integrity was determined by the HOS test for both chilled and frozen semen. The integrity was greatly improved by GH addition in the extender in both semen forms compared to the control with 5mM and 7mM concentration. This result was also supported by Stradaioli et al., (2007); Ogata et al., (2015); and Ahmed et al., (2021) who also shows better semen viability and integrity with addition of glutathione in semen extenders of different species.

Among the three concentrations of GH, the 5mM GH showed better sperm motility where both 5mm and 7mM GH showed similar viability and normal sperm percentage in frozen semen. However, 5mM GH concentration scored better motility, viability followed by 3mM GH in chilled on days 2 and 6. 7mM GH had similar Functional integrity value with on days2, 4 and 6 in Chilled semen. Overall, 5mM GH concentration had a higher extender quality compared to the others in both semen preservation types. This result is similar to Ahmed et al., (2021) who also reported; a 4mM GH supplemented extender increases sperm motility, viability, and integrity in rabbit semen.

In average, the result revealed that JP buck had significantly great opportunity than BB buck to preserve (chilled and frozen) for its more voluminous production of semen ejaculation and updating the modern breeding technique (Artificial insemination) in goat.

## 5.3. Effect of Vitamin-C as an antioxidant in preserved goat semen extender

Ascorbic acid (Vitamin-C) is a well-known antioxidant that safeguards the life of spermatozoa by preventing oxidative damage to DNA and membranes. Ascorbic acid is thought to suppress oxidative species and preserve the cell membrane and acrosome of sperm cells, resulting in greater sperm quality with semen extension in both sperm preservation and fertilization (Padayatty et al., 2003).

Individual motility was altered by the semen extender in this investigation, and Vitamin-C appeared to increase sperm motility substantially when compared to the control treatment. In chilled semen, improvement was found significantly only on day 2 by 7mg Vit-C whereas on days 4 and 6 results were non-influenced. However, on frozen semen, the motility improvement was noticed greatly by 5mg and 7mg Vit-C compared to the control on days3, 10 and 20. Significant difference was also observed among the concentrations, especially 7mg vitamin-C concentration showed better results compared to 3mg and 5mg concentration. Previous studies also reported improved sperm motility with Vit-C extender in different species like ram (Azawi and Hussein, 2013), bull (Foote et al., 2002; Hu et al., 2010; Achi et al., 2018; Singh and Sharma, 2018), and buffalo (Cheede et al., 2011) at the different times of preservation. Previous studies also suggested no effective alteration in sperm motility of stallion semen (Aurich et al., 1997; Ball et al., 2001). A few studies also reported a detrimental effect on sperm motility with high concentrations Vitamin-C in semen extenders of a frozen stallion (Alamaary et al., 2020) and bull semen (Beconi et al., 1993).

Vit-C semen extender had similar effect on sperm viability compared to control in chilled semen on days 2, 4 (except for day6 with 7mg vit-C), but it improved in frozen semen on days 3, 10, and 20 when compared to the control. In sperm morphology, the Vitamin-C addition had no significant effect on the normal sperm percentage in both frozen and chilled semen compared to the control. However, the addition of Vitamin-C to the extender considerably increased the integrity of sperm (HOS test value) on day 2 of chilled semen (by all concentrations) and on day 4 by only 7mg Vitamin-C. On the other hand, sperm integrity was not improved significantly except for 7mg Vitamin-C on day 3 and day 20 compared to the control. Like this result, the improvement of sperm viability and integrity with vit-C mixed extender had been shown by Aurich et al., (1997) in stallions, Yu et al., (2019) in donkey. But insignificant differences also had been reported by Michael et al., (2008) in dogs and Alamaary et al., (2020) in stallions.

**CHAPTER 6: CONCLUSION**

From the investigation, Jamunapari buck fresh semen has comparatively higher mass motility and sperm concentration compared to the Black Bangle goat while other semen parameters like pH, viability, and sperm functional integrity were uninfluenced. The study revealed that, semen quality was degrading significantly with advancing in storage time in chilled were remained uninfluenced in frozen state. Glutamine and Vitamin C as antioxidants were found to improve semen quality compare to the control in both chilled and frozen state.

As for Glutathione, sperm motility was improved significantly compared to the control treatment in both chilled and frozen forms on observed days. The sperm viability also improved with GH addition especially with 5mM in chilled semen as well as 5mM GH and 7mM GH on frozen semen. Normal sperm count was uninfluenced in chilled semen but significantly increased with only 5mM GH concentration on days 10 and 20 in frozen semen. Sperm functional integrity was greatly improved by GH addition in the extender in both semen forms compared to the control with 5mM and 7mM GH supplementation.

The Vit-C was improved sperm motility and viability significantly in frozen semen on days 3, 10, and 20 and day 2 in chilled semen. Vitamin-C considerably increased the functional integrity of sperm on days 2 and 4 of chilled semen and day3 and 20 of frozen semen especially with 7mg vit-C concentration while normal sperm percentage were un-influenced in both chilled semen and frozen semen.

In short, Glutathione and Vitamin-C improved the semen quality in both chilled and frozen form, especially with 5mM GH and 7mg vit-C concentrations, respectively. Although there was found not many significant differences between this two, but 5mM GH had slightly better values than 7mg vit-C as antioxidants supplementation.

**CHAPTER 7: LIMITATIONS AND RECOMMENDATIONS**

1. Further study can be done to improve the semen extender for preservation of buck semen with other extenders for comparison.
2. Evaluation of effects on preserved semen supplemented antioxidants in conception rate in goats.

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# APPENDIX

**Appendix-1: Comparative evaluation of fresh semen between Jumunapari and Black Bengal buck**

|  |  |  |  |
| --- | --- | --- | --- |
| **Semen**  **Parameters** | **Bucks** | | |
| **Jumunapari**  **n=18** | **Black Bengal**  **n=18** | **P value** |
| **Volume(ml)** | 1.05± 0.38a | 0.51±.12b | <0.001 |
| **PH** | 6.66±0.16a | 6.36±.15a | 0.06 |
| **Color** | Creamy | Creamy |  |
| **Density (0-5 score)** | 4.13 ± 0.30a | 4.37 ± 0.22b | 0.03 |
| **Mass motility**  **(0-5 score)** | 4.73 ± 0.45a | 4.41 ± 0.33a | 0.02 |
| **Concentration (x109 /ml)** | 3.50± 0.14a | 3.71±0.16b | 0.26 |
| **Live %** | 91.53± 4.5a | 91.84±4.8a | 0.39 |
| **Functional integrity (HOS test)** | 85.19± 4.08a | 85.08±4.20a | 0.95 |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Motility (%)** | **Extenders** | | **Preservation time (Day)** | | |
| **2** | **4** | **6** |
| TCEY-1 | Control | 78.88±1.73 | 73.28±1.34 | 57.08±2.36 |
| TCEY-2 | 3mM GH | 85.06±1.44 | 75.00±1.42 | 59.04±2.49 |
| 5mM GH | 86.83±1.56 | 79.53±1.61 | 62.14±2.08 |
| 7mM GH | 80.43±1.81 | 75.28±1.22 | 59.21±2.85 |
| TCEY-3 | 3mgVit-C | 81.04±0.33 | 74.90±1.77 | 57.33±1.33 |
| 5mgVit-C | 81.33±1.36 | 75.53±1.26 | 57.62±2.79 |
| 7mgVit-C | 84.17±1.19 | 75.59±1.42 | 58.56±2.98 |
| **Viability %(live)** | TCEY-1 | Control | 81.87±1.47 | 72.06±1.43 | 54.81±2.59 |
| TCEY-2 | 3mM GH | 84.59±1.42 | 75.88±1.83 | 63.06±2.21 |
| 5mM GH | 86.83±1.56 | 80.76±1.61 | 67.20±2.14 |
| 7mM GH | 83.96±1.70 | 76.02±1.02 | 59.22±3.14 |
| TCEY-3 | 3mgVit-C | 83.81±1.79 | 74.21±1.53 | 60.50±1.25 |
| 5mgVit-C | 84.37±2.10 | 75.24±1.96 | 61.05±2.79 |
| 7mgVit-C | 85.91±1.38 | 77.88±1.83 | 66.17±2.43 |
| **Normal sperm %** | TCEY-1 | Control | 96.00±1.38 | 95.63±1.38 | 95.33±1.45 |
| TCEY-2 | 3mM GH | 96.33±1.68 | 95.28±2.03 | 95.51±2.23 |
| 5mM GH | 96.93±2.40 | 95.69±2.83 | 96.48±1.73 |
| 7mM GH | 96.41±2.02 | 96.75±1.67 | 95.83±2.26 |
| TCEY-3 | 3mgVit-C | 95.73±1.65 | 95.40±2.85 | 96.78±1.44 |
| 5mgVit-C | 95.12±2.13 | 95.69±1.70 | 95.48±2.10 |
| 7mgVit-C | 96.09±1.91 | 94.92±2.37 | 96.48±0.48 |
| **Functional**  **intigrity testy(HOS test)%** | TCEY-1 | Control | 63.18±2.76 | 60.27±1.63 | 41.00±2.10 |
| TCEY-2 | 3mM GH | 73.79±2.47 | 62.08±2.15 | 45.28±2.15 |
| 5mM GH | 74.22±1.72 | 68.48±1.52 | 50.95±1.68 |
| 7mM GH | 70.00±1.57 | 68.13±2.06 | 48.46±2.12 |
| TCEY-3 | 3mgVit-C | 69.87±2.06 | 61.25±1.50 | 43.12±2.05 |
| 5mgVit-C | 70.17±1.35 | 63.83±1.76 | 42.01±2.00 |
| 7mgVit-C | 72.35±1.63 | 69.71±1.92 | 42.36±1.69 |

# Appendix-2: Effects of antioxidants Jumunapari buck chilled semen in three different preservation times

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Motility (%)** | **Extenders** | | **Preservation time (Day)** | | |
| **3** | **10** | **20** |
| TCEY-1 | Control | 41.71±0.09 | 40.55±1.88 | 40.05±1.11 |
| TCEY-2 | 3mM GH | 48.03±1.55 | 47.24±1.42 | 46.13±0.71 |
| 5mM GH | 52.33±0.14 | 52.25±3.98 | 51.18±2.56 |
| 7mM GH | 46.92±1.60 | 46.55±2.03 | 46.05±1.25 |
| TCEY-3 | 3mgVit-C | 43.44±2.07 | 42.17±3.60 | 40.71±1.04 |
| 5mgVit-C | 47.80±0.37 | 46.89±2.77 | 46.37±2.45 |
| 7mgVit-C | 51.38±0.96 | 50.63±2.93 | 50.51±2.53 |
| **Viability %(live)** | TCEY-1 | Control | 42.41±3.06 | 41.52±2.38 | 39.34±3.05 |
| TCEY-2 | 3mM GH | 48.91±4.88 | 48.52±3.97 | 47.15±3.73 |
| 5mM GH | 54.85±4.5 | 54.06±4.47 | 53.35±2.51 |
| 7mM GH | 52.00±7.84 | 51.12±4.81 | 51.52±1.32 |
| TCEY-3 | 3mgVit-C | 44.32±4.67 | 44.05±1.93 | 43.45±3.9 |
| 5mgVit-C | 47.14±3.50 | 45.49±2.7 | 45.09±2.82 |
| 7mgVit-C | 50.88±3.87 | 48.65±2.40 | 47.50±2.22 |
| **Normal sperm %** | TCEY-1 | Control | 94.02±1.88 | 93.52±1.87 | 90.85±1.65 |
| TCEY-2 | 3mM GH | 96.42±0.42 | 94.31±2.3 | 92.51±2.23 |
| 5mM GH | 98.98±1.54 | 97.33±1.84 | 97.05±1.25 |
| 7mM GH | 95.24±2.4 | 94.85±4.26 | 93.85±2.26 |
| TCEY-3 | 3mgVit-C | 95.16±3.24 | 95.06±2.06 | 93.23±1.73 |
| 5mgVit-C | 96.87±1.82 | 95.27±2.20 | 95.09±1.13 |
| 7mgVit-C | 96.59±2.19 | 96.66±1.68 | 94.21±2.36 |
| **Functional**  **intigrity testy(HOS test)%** | TCEY-1 | Control | 34.11±0.09 | 33.51±1.88 | 30.55±2.11 |
| TCEY-2 | 3mM GH | 36.97±3.12 | 35.97±3.27 | 34.81±3.01 |
| 5mM GH | 42.09±3.35 | 42.78±2.80 | 40.78±3.14 |
| 7mM GH | 40.98±2.84 | 39.24±2.54 | 39.08±1.34 |
| TCEY-3 | 3mgVit-C | 31.16±1.52 | 30.89±3.01 | 29.84±3.33 |
| 5mgVit-C | 34.88±2.21 | 34.63±0.90 | 32.27±2.03 |
| 7mgVit-C | 39.87±3.40 | 36.23±2.29 | 36.66±1.18 |

# Appendix 3: Effects of antioxidants Jumunapari buck frozen semen in three different preservation times

**BIOGRAPHY**

Dr. Omar Faruq is the son of Atar Ali and Ajiba Begum. He is a MS Student of Theriogenology under the Department of Medicine and Surgery at Chattogram Veterinary and Animal Sciences University (CVASU). He had successfully completed his DVM degree in 2020 from CVASU. At present he is working as a Customer Service Officer (CSO) of KAZI Farms Group. His research is based on the veterinary Andrology. He has a great interest on veterinary Andrology.