EFFECT OF BOVINE SERUM ALBUMIN ON SOME QUALITY PARAMETERS OF RAM CAUDA EPIDIDYMAL SPERMATOZOA SUBJECTED TO FREEZING-FREE PRESERVATION

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DECLARATION

I hereby declare that this work is the product of my research efforts undertaken under the supervision of Dr. A.M. Abdussamad and has not been presented anywhere for the award of degree or certificate. All sources have been duly acknowledged.

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CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent write-up (Ali Abdullahi Ahmad SPS/15/MBC/00046) were carried out under my supervision.

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This dissertation has been examined and approved for the award of Masters in (Biotechnology).

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ABSTRACT

The present study determined the effect of Bovine Serum Albumin (BSA), incubation and individual ram on total motility, intensity of motility, viability and DNA integrity of refrigerated (5^{o}C) epididymal spermatozoa. Cross sectional design and convenience sampling were used. One apparently healthy ram of ≥ 3 years of age was sampled per abattoir visit. Three weekly abattoir visits were made consecutively for six weeks. Total motility in yolk-citrate extender (positive control) was similar ($P>0.05$) to corresponding total motility in 1, 5, 10 and 15 % BSA-based extenders. Prior to 6 h incubation, intensity of motility in yolk-citrate extender was not significantly different with corresponding values in 0.3, 1, 5, 10 and 15 % BSA-based extenders. Spermatozoa in yolk-citrate extender had similar intensity of motility with those in 1, 5, 10 and 15 % BSA-based extenders after 6 h incubation. Viability of spermatozoa in 15 % BSA-based extender was significantly ($P<0.05$) higher than viability in yolk-citrate extender. However, DNA integrity of spermatozoa in yolk-citrate extender was similar to respective values in 1, 5, 10 and 15 % BSA-based extenders. Extender devoid of egg yolk and BSA (negative control) was significantly lower in terms of all quality parameters when compared to all extenders, except 0.3 % BSA-based extender which had similar intensity of motility with yolk-citrate extender after 6 h incubation. All quality parameters before incubation except DNA integrity were significantly ($P< 0.05$) higher than corresponding values after 6 h incubation. However, DNA integrity before and after incubation were similar. Significant individual differences in all quality parameters existed among rams. Similarly, significant interactive effect of incubation and individual ram on percent total motility and viability was recorded. In conclusion, BSA is a suitable replacement for egg yolk in ram epididymal spermatozoa preservation. However, incubation and individual differences are influencing factors that must be taken into consideration.

CHAPTER ONE

1.0 INTRODUCTION

1.1BACKGROUND INFORMATION

Genetic improvement of farm animals relies on the intensive use of a few superior males either for natural mating, or in artificial insemination programs. The production of meat and milk may be increased through selective breeding of ewes with rams exhibiting desirable genetic combinations (Abdel-Rahman *et al*., 2000). In livestock breeding, the preservation of gametes after the death of a genetically valuable animal can be important. Recovery of spermatozoa after death may offer the last chance to preserve genetic material from breeding animals which have died unexpectedly (García-Álvarez *et al*., 2009) and provide resources for the development of germplasm banks of an endangered species (Martinez-Pastor *et al*., 2005).

The interest in preserving endangered species and valuable genetic material has resulted in increased attention towards possible recovery of viable sperm from the epididymides of dead animals (Foote, 2000). Livestock industry plays a crucial part in livelihood system and economy of developing country. Advances in male and female germ cell preservation and transfer have developed rapidly in recent years. The goal of spermatozoa preservation is to extend the fertile life of genetically superior males so as to facilitate their effective use in assisted reproduction (Nang *et al*., 2011).

Bovine serum albumin (BSA) is a kind of serum albumin which could protect sperm cell efficiently added in semen extender (Matsuoka *et al*., 2006; El-Kon *et al*., 2011; Osman *et al*., 2012). Discovery of the use of BSA as sperm protective agent had begun since 1976. Blank *et al.* (1976) discovered that BSA can be adsorbed onto the surface of spermatozoa plasma membrane, thus protecting spermatozoa from harmful effects during preservation. Furthermore, Fukui *et al*. (2007) had proved that BSA can

help in improving the survival and fertility of cryopreserved spermatozoa. In both of these studies, BSA was used in place of egg yolk in the preservation extender. The spermatozoa preserved with BSA extender maintained their fertility properties which lead to successful fertilization (Nang *et al*., 2011).

Freezing-free preservation has been established to be able to lessen damage to spermatozoa compared to a freezing protocol (Osman *et al*., 2012). Even though freezing-free preservation can be used to inseminate (*in vivo*) in the production of normal offspring, duration of preservation using this preservation method has to be improved because it only allows short-term storage. This is supported by the fact that unfrozen spermatozoon can fertilize an oocyte after seven or eight days' storage based on the media used (Osman *et al*., 2012). However, all new extenders or preservation methods for semen need to be tested before practical application in the field. Therefore, *in vitro* studies to test the effectiveness of these methods are needed (Amann, 1989)

1.2 RESEARCH PROBLEM

Cryopreservation is by far a standard technique for the long-term preservation of spermatozoa (Sitaula *et al*., 2009). Nevertheless, a number of handling and logistical difficulties halted this technique from being carried out at most of the remote farms (Nang *et al*., 2011). The necessity of storage in liquid nitrogen (Meyers, 2006) and transportation difficulties of semen sample from laboratory to the farm or over the countries further complicate the use of this preservation technique (Nang *et al*., 2011). Preservation of sperm cells can be compromised if testis-epididymis samples are poorly packaged or stored due to post-mortem tissue degeneration (Sankai *et al*., 2001). Furthermore, time constraint is consistently identified as a major problem for

recovering motile and viable epididymal sperm cells from the abattoir-derived epididymides (Kikuchi *et al*., 1998; Chaveiro *et al*., 2015).

Indiscriminate slaughtered of an indigenous allow males would deplete the excellent genetic resources (Osamede and Adebowale, 2016). This poses the danger of losing valuable genes for adaptation to extreme environments and disease which are of value in developing countries (Osamede and Adebowale, 2016).

Improvement in this field can be done by discovering ideal storage temperature as well as supplementation with a compound that can maintain and improve quality of stored spermatozoa (Osman *et al*., 2012).

Ram spermatozoa have a special composition of cell membrane that makes them difficult to cryopreserved (Aisen *et al*., 2002). Ram sperm has a lower phospholipid to cholesterol ratio and higher fatty acids to polyunsaturated fatty acids ratio than other species (Çoyan *et al*., 2011). Sperm susceptibility to cryopreservation is explained by membrane phospholipids ratio and membrane cholesterol to phospholipids composition (Holt, 2000). Besides, changing lipid structure of sperm membrane affects sperm freezing (Amorim *et al*., 2009). It means that sperms having low cholesterol to phospholipid ratio as seen in boars, stallions, rams and bulls are more sensitive to the cryodamage than sperms from rabbits and humans which have high cholesterol to phospholipid ratios (Parks and Lynch, 1992; White, 1993).

1.3 JUSTIFICATION

The goal of spermatozoa preservation is to extend the fertile life of genetically superior males so as to facilitate their effective use in the field of assisted reproduction (Nang *et al*., 2011). Apart from handling and logistical difficulties, freezing of spermatozoa cause serious damage and impairs fertility of the

spermatozoa population (Nang *et al*., 2011). These damages are less pronounced in semen that had been preserved above freezing point (Maxwell & Salamon, 1993).

Instead of the conventional method of semen collection, epididymal sperm is rather collected to reduce costs and offset some organizational difficulties (Abu *et al*., 2016). This becomes necessary when natural mating or the use of ejaculated semen is not possible due to difficulty of handling intractable animal or sudden death of an animal (Homeida *et al*., 2001).

Bovine serum albumin (BSA), a highly soluble protein, naturally occurs in mammalian semen and protects the sperm cell from harmful effects of free radicals during oxidative stress (Fukuzawa *et al*., 2005; Roche *et al*., 2008). Supplementation of BSA in extender did not improve the kinetic parameters of spermatozoa during storage of equine semen (Ball *et al*., 2001). Contrary to this finding, studies in bovine (Uysal *et al*., 2007), caprine (Amidi *et al*., 2010; Anghel *et al*., 2010), equine (Kreider *et al*., 1985; Klem *et al*., 1986), ovine (Uysal and Bucak, 2007) and lapine semen (Alvarez and Storey, 1983) reported improvement in sperm motility and characteristics through BSA inclusion in extenders. However, the exact mechanism through which BSA stimulates motility is unknown (Harrison *et al*., 1982; Klem *et al*., 1986).

1.4 AIM AND OBJECTIVES OF THE STUDY

The aim of the study was to evaluate the effect of BSA on some quality parameters of ram cauda epididymal spermatozoa under freezing-free preservation. The specific objectives were:

i. To determine the effect of BSA on total motility, intensity of motility, membrane viability and DNA integrity of ram cauda epididymal spermatozoa.

ii. To compare total motility, intensity of motility, membrane viability and DNA integrity before 6 hour and after 6 hours incubation of ram cauda epididymal spermatozoa.

iii. To determine the effect of individual ram on total motility, intensity of motility, membrane viability and DNA integrity of ram cauda epididymal spermatozoa.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 POPULATION AND DISTRIBUTION OF SHEEP (*Ovis aries*)

According to the Food and Agriculture Organization (FAOSTAT, 2017) the global sheep population is more than 1.2 billion heads. The three largest destinations for sheep are Asia, Africa and Europe with 41.4 %, 28 % and 12 %, respectively (FAOSTAT, 2017). Nigeria is home to about 42.5 million sheep and about 70 % of the small ruminants are found in the semi-arid zones of Nigeria and these belong to the agro-pastoral farmers utilizing extensive and semi-intensive management systems (Ajala *et al*., 2003; Mbilu, 2007). According to Adu and Ngere (1979) and Ugwu (2007), the indigenous sheep breeds of northern Nigeria are the Yankasa, Ouda, Balami and their crosses. These breeds generally have heavier birth and adult weights, and grow faster than the West African Dwarf breed which thrives best in the lower fringes of the derived Savannah, and in the rainforest regions of southern Nigeria (Ugwu, 2007). The productivity of the sheep in the north is severely limited by the semi-arid and arid environments in which they live. Majority of the sheep population in the country are owned by small-holder rural livestock farmers, a few are still in the urban areas (Sanni *et al*., 2004; Mbilu, 2007). Sheep and goats constitute a good source of family income and livelihood, assets and agricultural resource for small holder farmers (Iyayi and Tona, 2004; Salem-Ben and Smith, 2008; Shittu *et al*., 2008). This again underlines the valuable contribution of small ruminants as income generating assets among small-holder livestock farmers (Mbilu, 2007; Shittu *et al*., 2008). They are kept mainly as a secondary investment and require minimal input.

2.2 SOCIO-ECONOMIC IMPORTANCE OF SHEEP (*Ovis aries*)

Integration of sheep with crop agriculture usually occurs under subsistence conditions. They form an integral part of the system, providing milk, meat, manure and cash to the farming family during the time of need. Sheep and goats are efficiently reared on marginal lands and are good users of crop residues (Fakoya, 2007; Sanni *et al*., 2004). As such, they provide the only practical means of using vast areas of natural grasslands in regions where crop production is almost impracticable (Ngatazie, 1989; Rege, 1993). Small ruminants have been reported to be prolific (Otchere, 1986) and need only short gestation periods to increase flock size. This, therefore, makes traditional small ruminant production system a low input but high output enterprise with predictable profitability and economic returns (Nwafor, 2004).

Sheep contribute enormously to the protein requirements of most developing countries (Mandal *et al*., 2007; Muhammad *et al*., 2008). In sub-Saharan Africa, sheep provide almost 30 % of the meat consumed and around 16 % of the milk produced. David-West (1985) estimated that sheep and goats contribute about 35 % of the total animal meat production in Nigeria. This ranks small ruminants as the second most important suppliers of meat protein to the population after cattle (Maigandi, 2001; Ajala *et al*., 2003; Ugwu, 2004). Despite the enormous contributions of the small holder farmer to Nigeria's livestock economy and development programme, and in spite of the special attributes possessed by small ruminants, the productivity potential of these animals is yet to be fully exploited (Maigandi, 2001; Aye, 2004; Magaji, 2004). Some of these productivity attributes include the ability of small ruminants to highly adapt to a broad range of environments utilizing a wide variety of plant species (Aye, 2004; Ugwu, 2004; Nwafor, 2004), as well as not being prone to high feed competition with other species like cattle and camels (Rege, 1993; Gatenby, 2002).

Due to their short generation time (gestation period) and high fecundity (Otchere, 1986), sheep are generally known to have high production efficiency. During periods of unpredictable food shortage, sheep have proven very useful to human beings in the supply of meat and milk products (Gatenby, 2002; Iyayi and Tona, 2004).

2.3 APPLICATION OF ASSISTED REPRODUCTIVE TECHNOLOGY

Assisted reproductive technologies such as artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer (ET) and cryopreservation of gametes allows exchange of genetic material between populations without the need to transport animals. It also eliminates problems of behavioral incompatibility, overcomes physical conditions that limit breeding, and reduces opportunities for disease transmission (Wildt, 1990; Loskutoff, 2003). Artificial insemination (AI) with frozen-thawed semen has been proposed as a valuable tool for genetic improvement programme for sheep (Anel *et al*., 2006). However, artificial insemination technique has still required a widespread application, because of very variable and frequently low fertility with cervical AI, forcing the use of short-term refrigerated semen or laparoscopic insemination. The main problem with the cervical AI is the difficulty in performing a deep insemination, because of the sheep reproductive tract anatomy and to the convoluted shape of the cervical channel, forcing to deposit the semen within the vagina or to perform shallow intracervical inseminations (Kaabi *et al*., 2006; Druart *et al*., 2009). Moreover, cryopreservation impairs sperm quality (Salamon and Maxwell, 2000), and possibly its ability to migrate to the oviduct, which explains the requirement of laparoscopic AI to achieve acceptable results when using cryopreserved semen in sheep.

The sheep industry has not been able to utilize many of the assisted reproductive technologies (ART) in general and AI in particular, as other livestock

industries, due to inefficiencies in collecting, freezing and inseminating frozen ram semen. Furthermore, some studies routinely collect and freeze ram semen, and there is still a need to optimize cryopreservation and breeding protocols for ram semen (Blackburn, 2004). Cryopreservation is an extensively used technique for the long-term storage of semen, but it causes partial irreversible damage to the sperm cells (Amann, 1989; Purdy, 2006).This has been attributed to sperm cold shock, oxidative stress, sperm membrane modification, cryoprotectant toxicity, intracellular ice crystal formation, and fluctuations in osmotic pressure (Watson and Martin, 1975; Watson, 1995; Isachenko, 2003).

Genetic improvement of farm animals relies on the intensive use of a few superior males either for natural mating, or in artificial insemination programs. The production of meat and milk may be increased through selective breeding of ewes with rams exhibiting desirable genetic combinations (Abdel-Rahman *et al*., 2000). However, sperm density and sperm membrane viability as well as other sexual characteristics of the different breeds may limit the extent to which rams can be used for breeding. The influence of the major biologically active inorganic components such as selenium on these sperm parameters has been extensively studied in various breeds of rams (Scott *et al*., 1963; Dott and White, 1964; Wallace and Wales, 1964; Quinn *et al*., 1965; Quinn and White, 1966; Stechell, 1974; Hamamah and Gatti, 1998).

2.4 EPIDIDYMIS

The epididymis constitutes an important part of the male genital duct system. It plays a key role in the maturation and storage of spermatozoa (Bedford, 1967). The epididymis is a highly convoluted tubule which connects the testis to the ductus deferens and is an important segment of the excurrent duct system of the testes that

performs a variety of functions (Beu *et al*., 2009). Various studies on mammalian epididymis have shown that it can be divided into distinct regions according to the biochemical, morphological and morphometric characteristics of its segments (Beguelini *et al*., 2010). Various divisions have been proposed and the most widely used is that dividing the organ into the initial segment, caput, corpus and cauda epididymis (Serre and Robaire, 1998). The vas deferens is the tubular structure which conducts spermatozoa from the epididymis to the urethra. After crossing the ureter in the abdominal cavity, it dilates into a spindle shaped enlargement, the ampulla (Archana *et al*., 2008). Comparative anatomical and histological studies on epididymis and vas deferens of local domestic ruminants are not available, except few references that are available in the textbook on ruminants in general (Sisson and Grossman, 1975; Dyce *et al*., 2002). The ability of the epididymis to protect spermatozoa from oxidative attack while stored at this site, through the local actions of antioxidants, has not thus far been systematically studied. The human epididymis contributes to the antioxidant capacity of seminal plasma and possesses region-specific antioxidant activity, which may potentially protect spermatozoa from oxidative attack during storage (Potts *et al*., 1999).

2.5 EPIDIDYMAL SPERMATOZOA

Epididymal sperm has been used in many laboratories because it is easier to get in some special species (Tajik and Hassan-Nejad, 2008). Cryopreserved epididymal sperm is now used for intra-cytoplasmic sperm injection (ICSI) in human insemination (Jansen *et al*., 2000). Epididymal sperms have been obtained and individual variations in cryoprotectant toxicities have been studied for African antelope (Loskutoff *et al*., 1996). Epididymal sperms have successfully been obtained at necropsy from goats and used for *in vitro* fertilization (IVF) (Blash *et al*., 2000).

One year later, goat epididymal sperm was cryopreserved using a chemically defined model system (Kundu *et al*., 2001). Yu and Leibo (2002) have successfully recovered motile and membrane-intact spermatozoa from canine epididymis stored for 8 days at 4 °C. Similarly, James *et al*. (2002) have stored equine sperm in the epididymis at 4 °C for 24, 48, 72 and 96 hours. Some experiments were carried out by Kaabi *et al*. (2003) on the quality of cauda ram epididymal spermatozoa. On the other hand, artificial insemination and embryo transfer as well as IVF have been used in camelids (Roberts, 1991; Anouassi *et al*., 1992; Musa *et al*., 1992; McKinnon *et al*., 1994; Bravo *et al*., 2000). Surprisingly, some researchers have used epididymal sperm from South American camelids but no offspring resulted from their works (Del Campo *et al*., 1994).

Epididymal spermatozoa have been preserved or used not only in domestic animals but also in primates, rodents, experimental animals and humans for artificial insemination and IVF (Paŭfler and Foote, 1968; Foote and Igboeli, 1968; Marks *et al*., 1994; Blash *et al*., 2000; Morris *et al*., 2002; Morton *et al*., 2007; Morton *et al*., 2010).

2.6 SEMEN EXTENDER

Extender or diluent is a chemical medium used for preservation, extension and protection of sperm cells against various shocks during processing, storage and transportation used for artificial insemination (Nitin *et al*., 2018). Good extender should provide energy for metabolic activities within sperm cell; maintain osmotic pressure and pH of the medium (Salamon and Maxwell, 2000). Extender also keeps a check on the contamination of the medium to protect semen from microbial growth (Rehman *et al*., 2013). Different semen extenders provide sufficient nutrition in the form of fructose sugar to sperm cells during storage. It also prevents sperm cells

against cryoshocks during cold storage at extreme temperature (-196^{o}C) in liquid nitrogen (Foote, 2002). Moreover, liquid extended semen produces a higher conception rate with a relatively a smaller number of sperm cells (Shahab and Ahmad, 2003). Semen extender is the only medium which enables us to exploit the reproductive potential of the male animal more efficiently, with almost no venereal diseases (Rehman *et al*., 2013).

Different extenders have been designed and used in cattle (Vishwanath and Shannon, 2000), sheep (Paulenz *et al*., 2002), swine (Zou and Yang, 2000), canines (Iguer-ouada and Verstegen, 2001), deer (Asher *et al*., 2000), elephant (Graham *et al*., 2004) and even bactrian camels (Chen *et al*., 1990) to protect and maintain spermatozoa during processing and storage. AI with fresh and frozen semen is used for breeding bactrian camels routinely (Chen *et al*., 1990) with a high conception rate, whereas in dromedaries, attempts have been made to collect and use semen for AI, extended in different diluents, with variable results (Abdel-Raouf and El-Naggar, 1976; Taha-Ismail, 1988; Sieme *et al*., 1990; Billah and Skidmore, 1992; Musa *et al*., 1992). There is need for studying different types of extenders and storage conditions for the ejaculated spermatozoa in different species in order to develop the technique of AI or IVF and ET. During preservation of spermatozoa focusing on the quality parameters such as sperm viability, progressive motility, membrane integrity and acrosomal status are considered to be most reliable (Amann, 1989).

2.7 ROLE OF ANTIOXIDANTS

Antioxidants are the main defense factors against oxidative stress induced by free radicals (Silva *et al*., 2011). Under normal conditions, to neutralize the detrimental effects of reactive oxygen species (ROS) on spermatozoa, seminal plasma possess a number of natural antioxidant systems that scavenge ROS and prevent

internal cellular damage (Gadea *et al*., 2011). The imbalance between the presence of ROS and sperm antioxidant activity is the main cause of cryodamage of sperm (Wang *et al*., 1997; Ball, 2008; Li *et al*., 2010). The specific structures of spermatozoa are the plasma membrane, a large number of mitochondria, low cytoplasm and low antioxidant content in sperm cell cytoplasm which makes them possibly vulnerable to the damage of free radicals (Bollwein *et al*., 2008).

Antioxidants exert a protective effect on the plasma membrane of frozen bovine sperm, preserving both metabolic activity and cellular viability (Beconi *et al*., 1993). Although a significant negative correlation between the ROS level and the *in vitro* fertilization rate has been found (Agarwal *et al*., 2005), controlled quantities of ROS have been shown to be essential for the development and hyperactivation of spermatozoa (De Lamirande and Gagnon, 1993), two physiological processes of the spermatozoon that are necessary to ensure fertilization. The maintenance of a suitable ROS level is, therefore, essential for adequate sperm functionality. The presence of antioxidant enzymes such as glutathione reductase (GR), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), in bull (Beconi *et al*., 1993) and ram (Abu-Erreish *et al*., 1978; Marti *et al*., 2003; Marti *et al*., 2007) semen, and the effect of semen dilution in reducing their protective capacity (Maxwell and Stojanov, 1996) have been shown.

2.8 TYPES OF ANTIOXIDANTS

There are two types of antioxidants which include enzymatic antioxidants and non-enzymatic antioxidants (Kefer *et al*., 2009). Enzymatic antioxidants, also known as natural antioxidants include GPx, GR, SOD and CAT (Alvarez *et al*., 1987; Alvarez and Storey, 1989), all participate in sperm natural antioxidant defense system (Partyka *et al*., 2012). Non-enzymatic antioxidants, also known as synthetic

antioxidants or dietary supplements, include reduced glutathione (GSH), urate, ascorbic acid, vitamin E (alpha-tocopherol), carotenoids (beta-carotene), ubiquinones, taurine and hypotaurine, selenium and zinc (Alvarez and Storey, 1989; Therond *et al*., 1996).

Bovine serum albumin (BSA) is known to improve sperm motility, maintain plasma membrane integrity and protect acrosome reaction against temperature shock during the freeze–thaw process in ram semen (Uysal and Bucak, 2007). It may also enhance spermartozoa survival in the reproductive tract of the cow prior to fertilization (Chen *et al*., 1993). Besides, it was reported that BSA gives best fertility rates and increases the activity of catalase antioxidant activity following the freeze-thaw process in bull semen (Schafer and Holzmann, 2000). BSA increases motility and viability of spermatozoa after long term storage in low temperatures (Matsuoka *et al*., 2006; Yoshimoto *et al*., 2008) and during cryopreservation (Blesbois and Caffin, 1992). Also, a good membrane protection in terms of resistance to hypo-osmotic shock was also attained when BSA and egg yolk were added to the extender (Amidi *et al*., 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 SAMPLING LOCATION

Samples of intact ram scrotum containing the testes and epididymides were collected in early dry season September-October from the Kano Main abattoir located at Kofar Mazugal within Kano Metropolis in Dala Local Government Area of Kano State with the following GPS coordinates: 12.0128^0 N, 08.5211^0 E. Different animals which include camels, cattle, sheep and goats are slaughtered daily in the abattoir.

3.2 SAMPLING METHOD AND NUMBER OF RAMS SAMPLED

Using the convenience sampling method one (1) apparently healthy adult ram was selected. Age of ram was estimated by the dentition method of Johnson (1999) and only rams $\geq$ 3 years of age were considered. Three visits were undertaken weekly making a total of three rams per week and 18 rams in six weeks.

3.3 ABATTOIR PHASE

Upon slaughter and evisceration, the entire scrotum containing testes and attached epididymides was detached from the body wall using a sharp knife. It was placed on ice in a Styrofoam box and transported to the laboratory situated at the Centre for Dryland Agriculture, Bayero University, Kano (New Site) for the evaluation of spermatozoa quality parameters. This procedure was repeated throughout the sample collection period of six (6) weeks.

3.4 LABORATORY PHASE

3.4.1 PREPARATION BSA STOCK SOLUTION

BSA stock solution was prepared by dissolving 800 mg of BSA crystalline powder (Kem Light Laboratory Pvt. Ltd., Mumbai, India) into 100 ml of physiological saline (Dana Pharmaceutical Ltd, Minna, Niger State, Nigeria).

3.4.2 EXTENDER COMPOSITION

Seven different extenders were prepared: Extenders I and II were the positive and negative controls, respectively, while the remaining extenders (III-VII) were BSA-based as presented in Table 3.1 below.

Table 3.1 Composition of Test Extenders

Component	**Extender**						
	I	II	III	IV	V	VI	VII
Tri-sodium citrate, (g)	2.9	2.9	2.9	2.9	2.9	2.9	2.9
Egg yolk, (ml)	20	-	-	-	-	-	-
Sodium penicillin G, (IU)	100,000	100,000	100,000	100,000	100,000	100,000	100,000
Streptomycin sulphate, (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Distilled water, (ml)	To 100	To 100	To 100	To 100	To 100	To 100	To 100
BSA, % (v/v)	-	-	0.3	1	5	10	15
Fructose, (g)	1.250	1.250	1.250	1.250	1.250	1.250	1.250

I = Positive Control; II = Negative Control; III-VII = BSA-based Extenders

3.4.3 RECOVERY OF EPIDIDYMAL SPERMATOZOA

On arrival at the laboratory, the scrotum was dissected using scalpel blade and sharp scissors. The testis with its associated epididymis was removed from the tunica vaginalis. The epididymis was detached from the wall of the testis. It was divided into head, body and tail regions. The tail region was immersed into 40 ml of physiological saline. It was cut into pieces while immersed in the physiological saline and allowed to stand for 5 minutes, after which the content was agitated gently and filtered through a soft tissue paper. The filtrate was collected into a beaker. The same procedure was

followed for the remaining pairs of epididymides harvested throughout the experiment.

3.5 SPERMATOZOA QUALITY PARAMETERS

To determine spermatozoa quality parameters, 1.25 ml of the filtrate was transferred into 5 ml of respective extenders. The mixture of filtrate and extender was assessed before and after six hours of incubation at 5 ^{o}C.

3.5.1 DETERMINATION OF TOTAL MOTILITY AND INTENSITY OF MOTILITY

The total motility and intensity of motility were assessed as described by Abdussamad *et al*. (2015). Ten (10) µl of the mixture was placed on a glass slide and covered with a cover slip. The slide and cover slip were previously pre-warmed at 37 ^{o}C on a stage warmer. It was observed under light microscope at ×40 high power eye piece magnification. The total motility was recorded by taking the average of five fields and the intensity of motility of spermatozoa was scored as follows:

0 - No sperm movement.

1 - Slight tail undulation without forward motion.

2 - Slow tail undulation with slow or stop and start forward motion.

3 - Forward progression at a moderate speed.

4 - Rapid forward progression.

5 - Very rapid progression in which cells are difficult to follow visually.

3.5.2 DETERMINATION OF SPERMATOZOA VIABILITY

Viability was determined using Hypo-osmotic swelling test (HOST) as described by Jeyendran *et al*. (1984). Hypo-osmotic solution with osmolarity of 179 mOsmol/l was prepared by dissolving 1.375 g of D-Fructose and 0.75 g of sodium citrate in 100 ml of distilled water. One (1) ml of hypo-osmotic solution was mixed with 0.1ml of sample in a test tube and it was incubated at 37 ^{o}C for 30

minutes. A drop of the diluted sample was placed on a pre-warmed clean dry glass slide and covered with a cover slip. Spermatozoa were counted at x40 eye piece magnification in five different fields under light microscope. Live spermatozoa showed swelling of the tail which gives the appearance of a curled tail and dead spermatozoa had straight tail.

3.5.3 DETERMINATION OF DNA INTEGRITY

Spermatozoa DNA integrity was assessed using toluidine blue stain (Loba Chemie Pvt. Ltd., Mumbai, India) as described by Mello (1982) with modifications. Using 100 µl mixture of the filtrate and the extender, smears were made on glass slides, air dried, fixed in freshly prepared 96 % ethanol-acetone (1:1) at 4 ^{o}C for 10-15 min and hydrolyzed in 4.0 N HCl at 25 ^{o}C for 10-15 min. Slides were then rinsed three times in distilled water for 1 min each and stained with 0.05 % toluidine blue for 10 min. The staining buffer consisted of 50 % citrate phosphate (Mcllvain's buffer, pH 3.5). The slides were photographed in five different fields using microscope digital camera (AmScope, Irvine, CA 92606, USA) mounted on a trinocular microscope (Hund Wetzler, Model H600, Helmut Hund GmbH, Germany). The images were evaluated for DNA integrity. Sperm heads with intact chromatin (intact DNA) stained light blue and those with damaged chromatin (damaged DNA) stained violet or purple.

3.6 STATISTICAL ANALYSIS

Data were analyzed by a Three-Way Analysis of Variance (ANOVA) with extender and ram as between-subject variables and incubation as within-subject variable. The General Linear Model (GLM) Repeated Measures procedure of SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used for the analysis. Where significant differences exist, Tukey's test was used in most cases. However, where ANOVA showed significant difference but Turkey's test revealed no statistical

significance; Duncan Multiple Range Test was used for mean separation. Data for intensity of motility were analyzed using Kruskal-Wallis and Wilcoxon-signed Rank tests as implemented in GraphPad Instat® package (GraphPad InStat®, version 3.05, 32 bit for Win 95/NT, GraphPad Software, Inc, 2000).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Effect of Six-Hour Incubation on Total Motility, Viability, DNA Integrity and Intensity of Motility of Ram Epididymal Spermatozoa

Percent total motility of ram epididymal spermatozoa as affected by six-hour incubation is presented in Figure 4.1. Total motility before 6 h incubation (Mean = 73 %, SEM = 1 %) was significantly ($P<0.001$) higher than after 6 h incubation (Mean = 68 %, SEM = 1 %).

Percent viability of ram epididymal spermatozoa as affected by six-hour incubation is shown in Figure 4.2. Viability before 6 h incubation (Mean = 68 %, SEM = 1 %) was significantly ($P<0.05$) higher than after 6 h incubation (Mean = 65 %, SEM = 1 %).

DNA integrity of ram epididymal spermatozoa as affected by six-hour incubation is highlighted in Figure 4.3. No significant ($P>0.05$) effect was recorded between DNA integrity before (Mean = 69 %, SEM = 1 %) and after (Mean = 68 %, SEM = 0.6 %) 6 h incubation.

Intensity of motility of ram epididymal spermatozoa as affected by six-hour incubation is depicted in Figure 4.4. Intensity of motility before 6 h incubation (Median = 5) was significantly ($P<0.0001$) higher than after 6 h incubation (Median = 4).

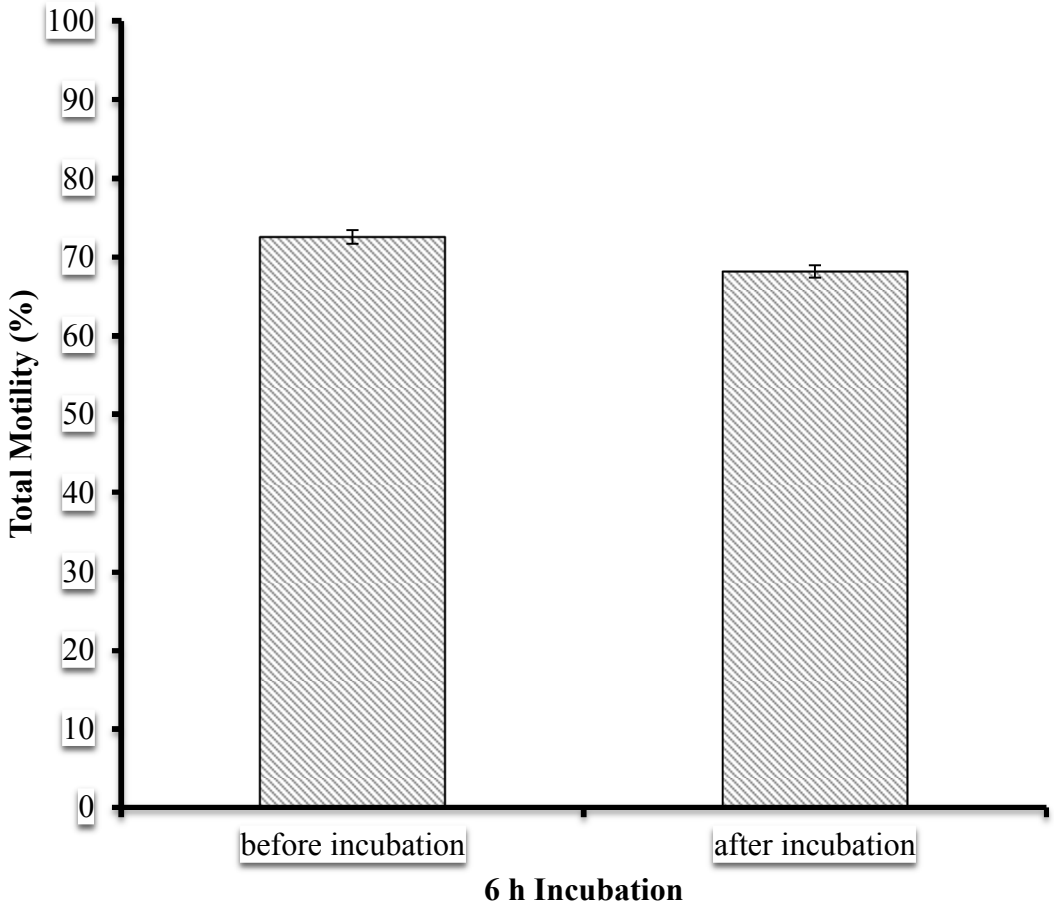

Figure 4.1 Percent Total Motility of Ram Epididymal Spermatozoa as Affected by Six-Hour Incubation

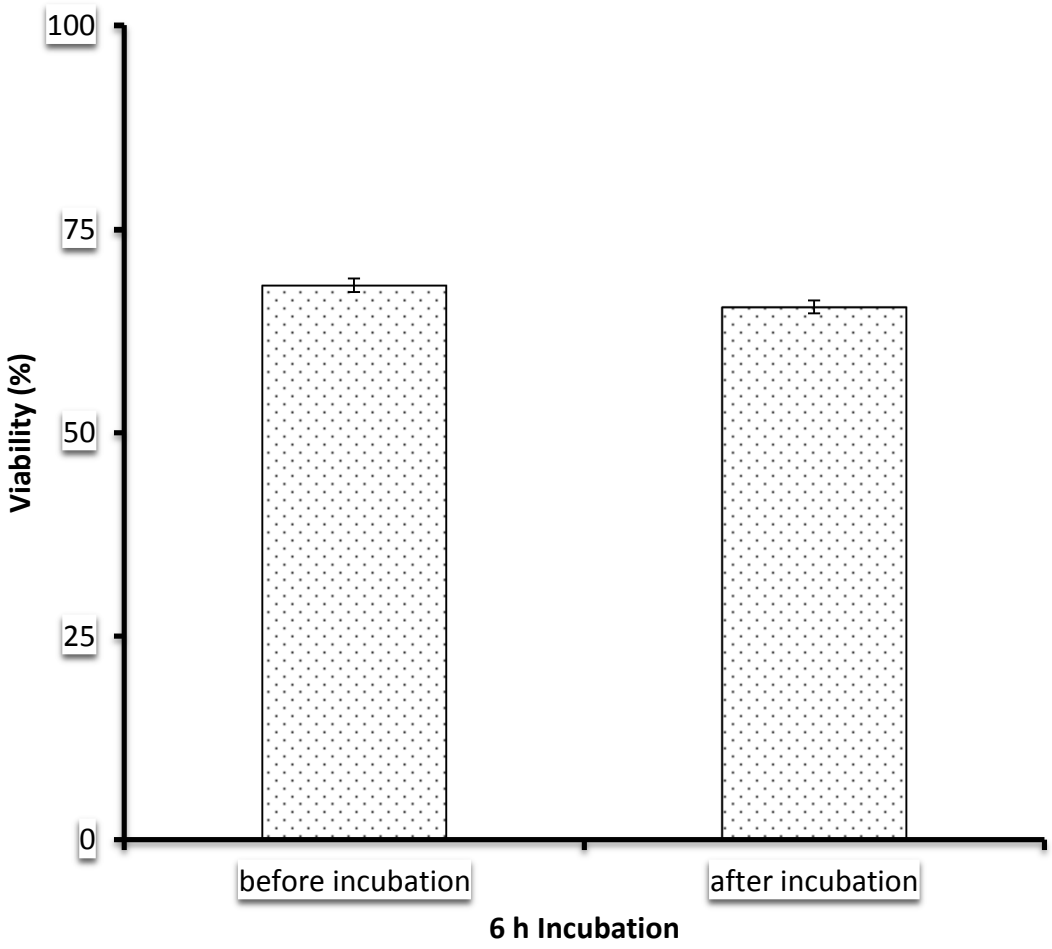

Figure 4.2 Percent Viability of Ram Epididymal Spermatozoa as Affected by Six-Hour Incubation

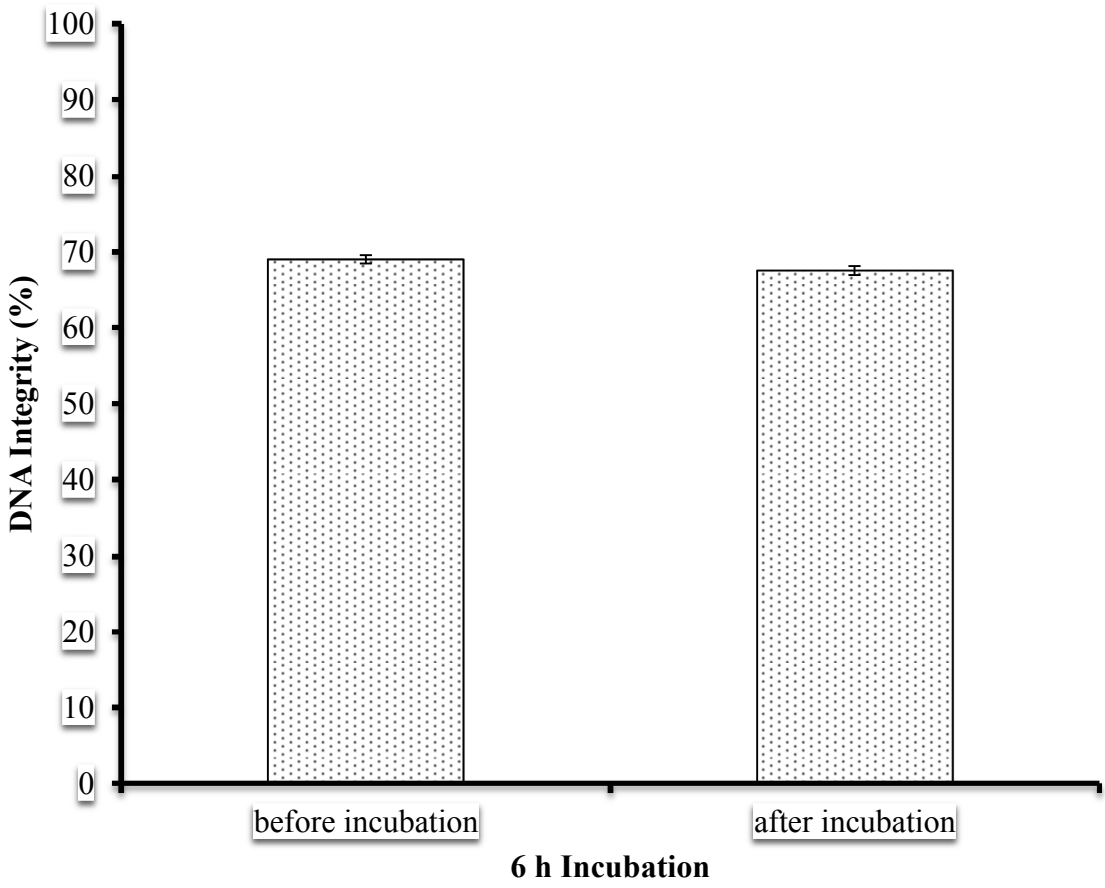

Figure 4.3 Percent DNA Integrity of Ram Epididymal Spermatozoa as Affected by Six-Hour Incubation

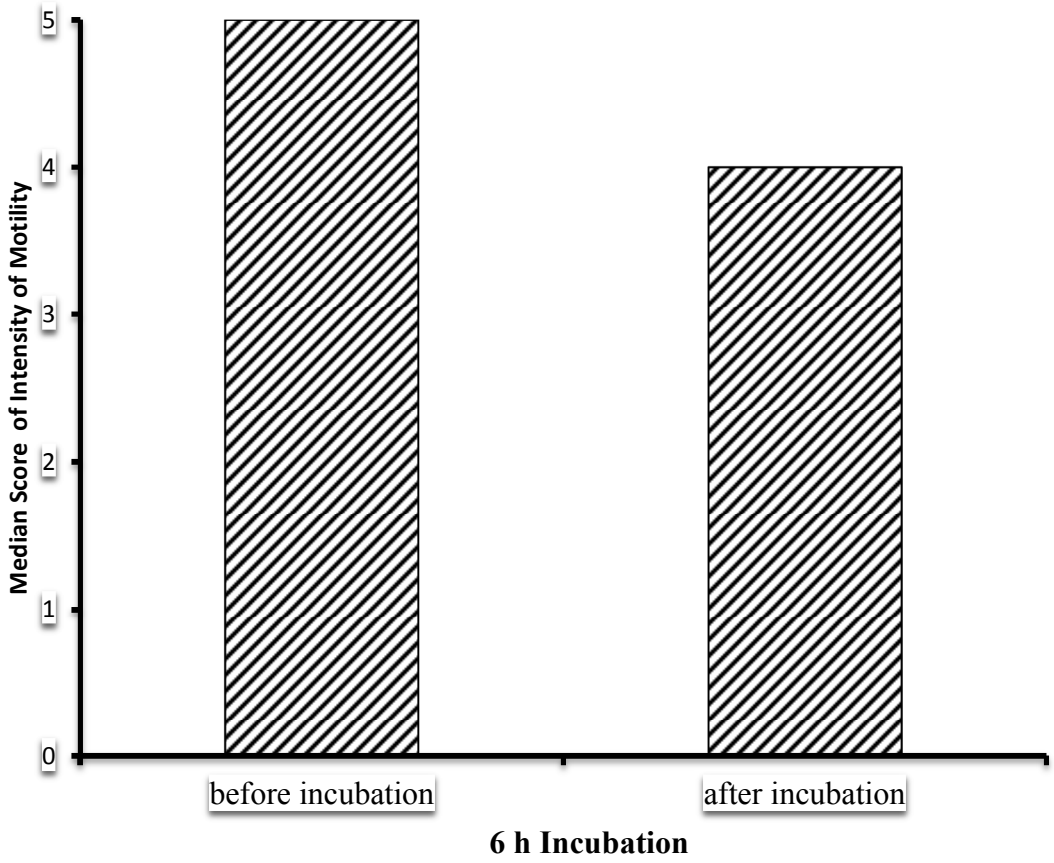

Figure 4.4 Intensity of Motility of Ram Epididymal Spermatozoa as Affected by Six-Hour Incubation.

4.1.2 Effect of Extender on Total Motility of Ram Epididymal Spermatozoa

Percent total motility of ram epididymal spermatozoa as affected by extender is shown in Figure 4.5. Total motility (77 ± 2 %) in yolk citrate extender (positive control) decreased significantly ($P<0.001$) by a difference of 21 % in extender devoid of BSA and egg yolk (negative control) with corresponding total motility value of 56 ± 2 %. Subsequently, total motility in the negative control extender increased significantly ($P<0.001$) by differences of 11, 14, 16, 19 and 21 % in 0.3, 1, 5, 10 and 15 % BSA-based extenders, respectively. Total motility (77 ± 2 %) in the positive control extender decreased significantly ($P<0.01$) by a difference of 10 % in 0.3% BSA-based extender. However, total motility (68 ± 2 %) in 0.3 % BSA-based extender increased significantly by differences of 8 ($P<0.05$) and 10 % ($P<0.01$) in 10 and 15 % BSA-based extenders, respectively. No significant ($P>0.05$) differences in total motility were observed in comparisons involving the following extenders: positive control *vs*. 1 % BSA, positive control *vs*. 5 % BSA, positive control *vs*. 10 % BSA, positive control *vs*. 15 % BSA, 0.3 % BSA *vs*. 1 % BSA, 0.3 % BSA *vs*. 5 % BSA, 1 % BSA *vs*. 5 % BSA, 1 % BSA *vs*. 10 % BSA, 1 % BSA *vs*. 15 % BSA, 5 % BSA *vs*. 0.3 % BSA, 5 % BSA *vs*. 1 % BSA, 5 % BSA *vs*. 10 % BSA, 5 % BSA *vs*. 15 % BSA, and 10 % BSA *vs*. 15 % BSA.

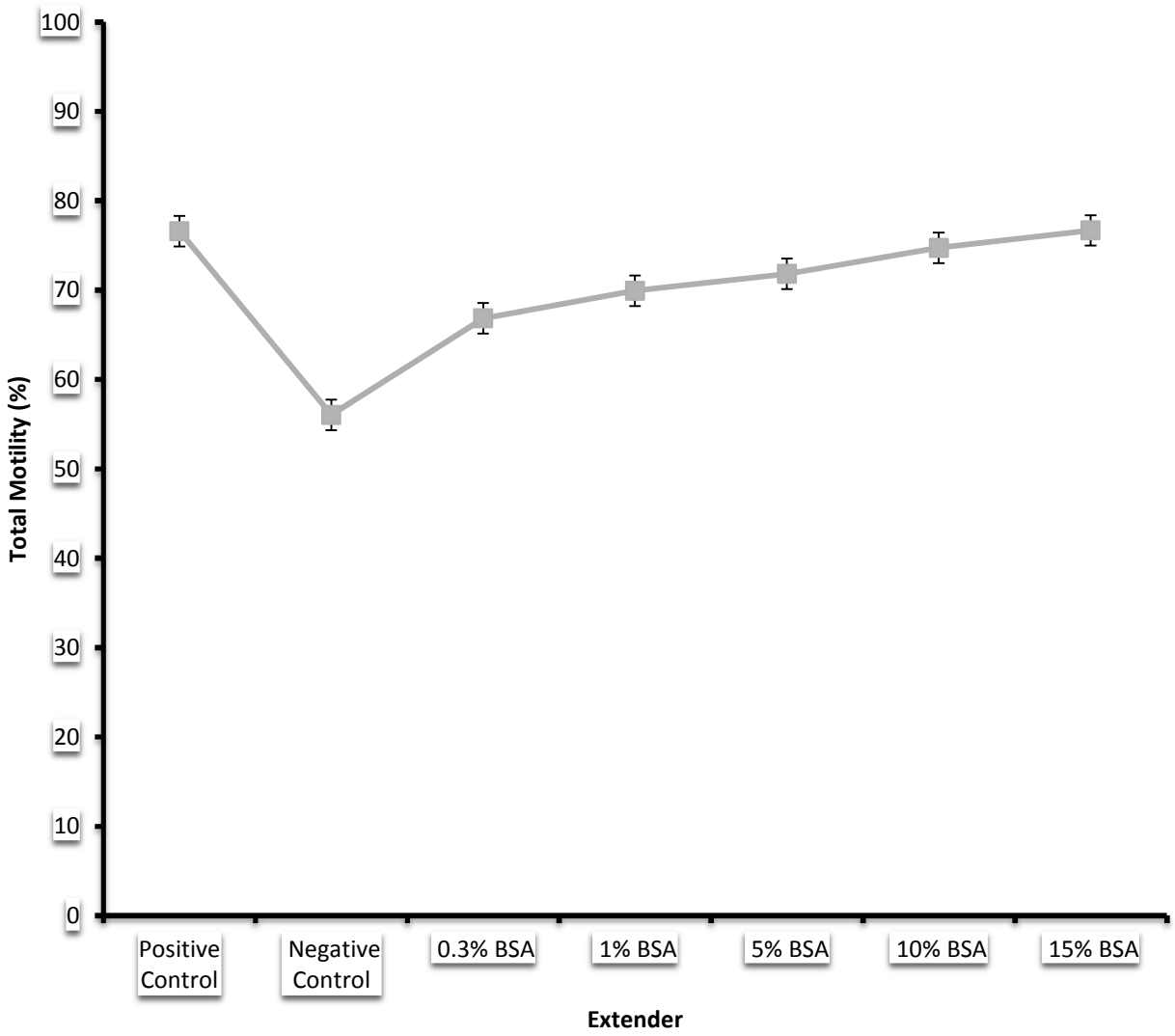

Figure 4.5 Percent Total Motility of Ram Epididymal Spermatozoa as Affected by Extender

4.1.3 Effect of Extender on Membrane Viability of Ram Epididymal Spermatozoa

Percent viability of ram epididymal spermatozoa as affected by extender is presented in Figure 4.6. Viability (68 ± 2 %) in yolk citrate extender (positive control) decreased significantly ($P<0.001$) by a differential of 21 % in extender devoid of BSA and egg yolk (negative control) with corresponding total motility value of 47 ± 2 %. Subsequently, viability in the negative control extender increased significantly ($P<0.001$) by differentials of 20, 23, 23, 25 and 29 % in 0.3, 1, 5, 10 and 15 % BSA-based extenders, respectively. Viability (68 ± 2 %) in the positive control extender increased significantly ($P<0.05$) by a differential of 8 % in 15 % BSA-based extender. Similarly, viability (67 ± 2 %) in 0.3 % BSA-based extender increased significantly ($P<0.01$) by a differential of 9 % in 15 % BSA-based extenders. No significant ($P>0.05$) differences in viability were observed in comparisons involving the following extenders: positive control *vs*. 0.3 % BSA, positive control *vs*. 1 % BSA, positive control *vs*. 5 % BSA, positive control *vs*. 10 % BSA, 0.3 % BSA *vs*. 1 % BSA, 0.3 % BSA *vs*. 5 % BSA, 0.3 % BSA *vs*. 10 % BSA, 1 % BSA *vs*. 0.3 % BSA, 1 % BSA *vs*. 5 % BSA, 1 % BSA *vs*. 10 % BSA, 1 % BSA *vs*. 15 % BSA, 5 % BSA *vs*. 0.3 % BSA, 5 % BSA *vs*. 1 % BSA, 5 % BSA *vs*. 10 % BSA, 5 % BSA *vs*. 15 % BSA, and 10 % BSA *vs*. 15 % BSA.

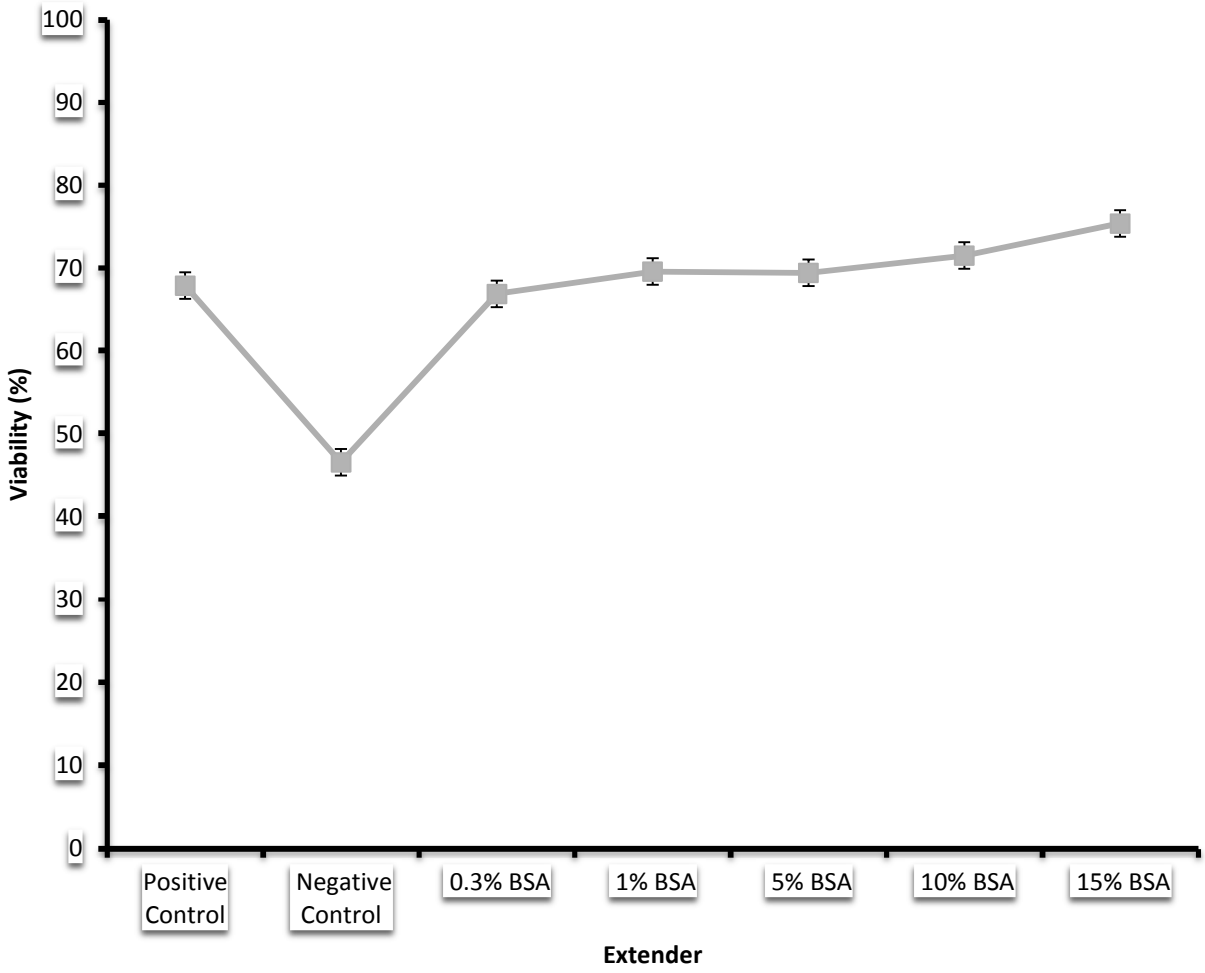

Figure 4.6 Percent Viability of Ram Epididymal Spermatozoa as Affected by Extender

4.1.4 Effect of Extender on DNA Integrity of Ram Epididymal Spermatozoa

DNA integrity of ram epididymal spermatozoa as affected by extender is highlighted in Figure 4.7. DNA integrity (73 ± 1 %) in yolk citrate extender (positive control) decreased significantly ($P<0.001$) by a difference of 25 % in extender devoid of BSA and egg yolk (negative control) with corresponding DNA integrity value of 48 ± 1 %. Subsequently, DNA integrity in the negative control extender increased significantly ($P<0.001$) by differences of 18, 22, 24, 27 and 28 % in 0.3, 1, 5, 10 and 15 % BSA-based extenders, respectively. DNA integrity (73 ± 1 %) in the positive control extender decreased significantly ($P<0.01$) by a difference of 7 % in 0.3 % BSA-based extender. However, DNA integrity (66 ± 1 %) in 0.3 % BSA-based extender increased significantly by differences of 6 ($P<0.05$), 9 ($P<0.001$) and 10 % ($P<0.001$) in 5, 10 and 15 % BSA-based extenders, respectively. Also, DNA integrity (69 ± 1 %) in 1 % BSA-based extender increased significantly by differences of 5 ($P<0.05$) and 6 % ($P<0.01$) in 10 and 15 % BSA-based extenders, respectively. No significant ($P>0.05$) differences in DNA integrity were observed in comparisons involving the following extenders: positive control *vs*. 1 % BSA, positive control *vs*. 5 % BSA, positive control *vs*. 10 % BSA, positive control *vs*. 15 % BSA, 0.3 % BSA *vs*. 1 % BSA, 1 % BSA *vs*. 5 % BSA, 5 % BSA *vs*. 10 % BSA, 5 % BSA *vs*. 15 % BSA, and 10 % BSA *vs*. 15 % BSA.

v

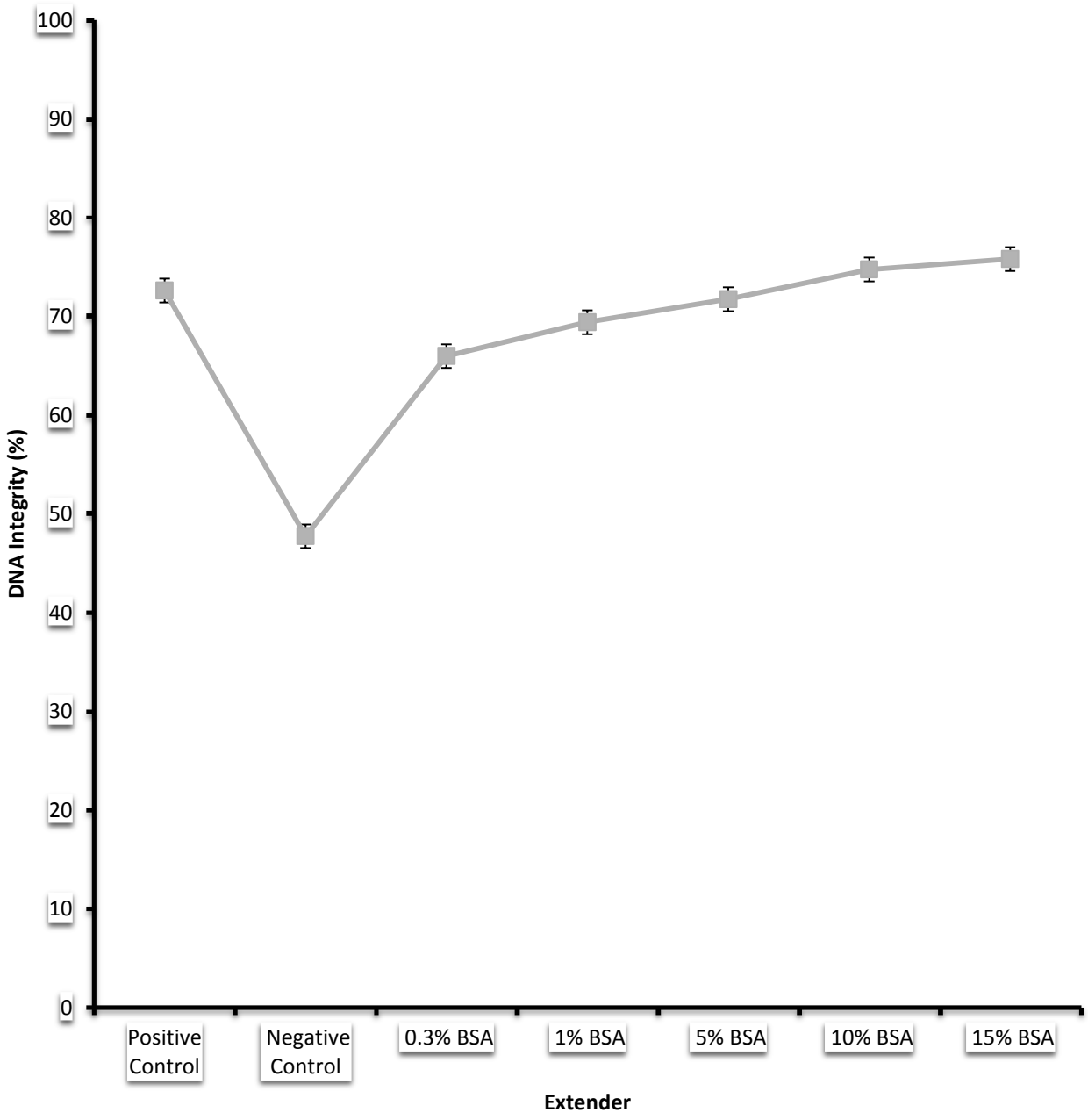

Figure 4.7 DNA Integrity of Ram Epididymal Spermatozoa as Affected by Extender

4.1.5 Effect of Extender on Intensity of Motility of Ram Epididymal Spermatozoa Before Six-Hour Incubation.

Intensity of motility of ram epididymal spermatozoa before six-hour incubation as affected by extender is depicted in Figure 4.8. Mean of ranks (78.333) of intensity of motility in yolk citrate extender (positive control) decreased significantly ($P<0.01$) by a difference of 36.944 in extender devoid of BSA and egg yolk (negative control) with corresponding mean of ranks of intensity of motility of 41.389. However, mean of ranks (41.389) of intensity of motility in negative control extender increased significantly ($P<0.01$) by a difference of 36.139 in 15 % BSA-based extender with corresponding mean of ranks of intensity of motility of 77.528. No significant ($P>0.05$) differences in mean of ranks of intensity of motility were observed in comparisons involving the following extenders: positive control *vs*. 0.3 % BSA, positive control *vs*. 1 % BSA, positive control *vs*. 5 % BSA, positive control *vs*. 10 % BSA, positive control *vs*. 15 % BSA, negative control *vs*. 0.3 % BSA, negative control *vs*. 1 % BSA, negative control *vs*. 5 % BSA, negative control *vs*. 10 % BSA, 0.3 % BSA *vs*. 1 % BSA, 0.3 % BSA *vs*. 5 % BSA, 0.3 % BSA *vs*. 10 % BSA, 0.3 % BSA *vs*. 15 % BSA, 1 % BSA *vs*. 5 % BSA, 1 % BSA *vs*. 10 % BSA, 1 % BSA *vs*. 15 % BSA, 5 % BSA *vs*. 10 % BSA, 5 % BSA *vs*. 15 % BSA, and 10 % BSA *vs*. 15 % BSA.

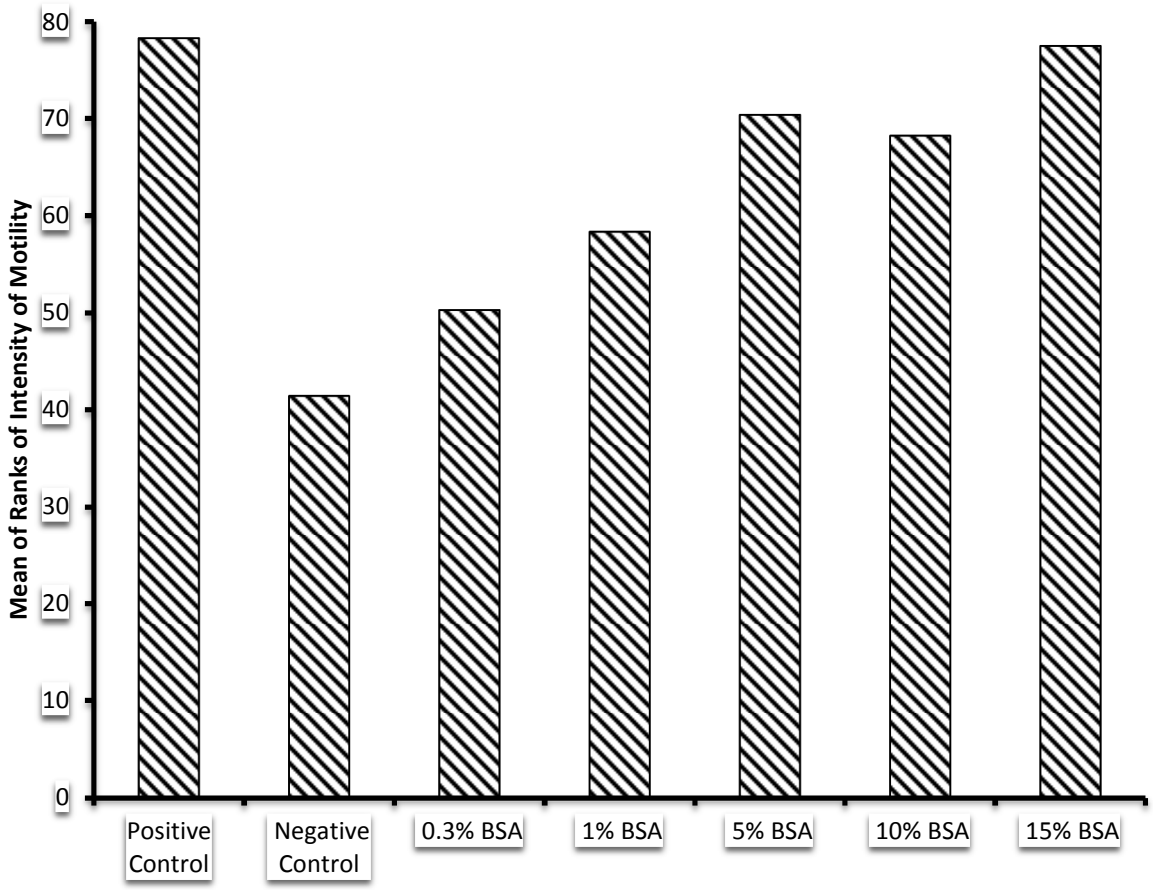

Figure 4.8 Intensity of Motility Before Six-Hour Incubation as Affected by Extender

4.1.6 Effect of Extender on Intensity of Motility of Ram Epididymal Spermatozoa After Six-Hour Incubation.

Intensity of motility of ram epididymal spermatozoa after six-hour incubation as affected by extender is depicted in Figure 4.9. Mean of ranks (91.694) of intensity of motility in yolk citrate extender (positive control) decreased significantly ($P<0.001$) by a differential of 52.944 in extender devoid of BSA and egg yolk (negative control) with corresponding mean of ranks of intensity of motility of 38.750. However, mean of ranks (38.750) of intensity of motility in negative control extender increased significantly ($P<0.01$) by a differential of 41.444 in 15 % BSA-based extender with corresponding mean of ranks of intensity of motility of 80.194. Similarly, mean of ranks (38.417) of intensity of motility in 0.3 % BSA-based extender increased significantly ($P<0.01$) by a differential of 41.777 in 15 % BSA-based extender. However, mean of ranks (91.694) of intensity of motility in the positive control extender decreased significantly ($P<0.001$) by a differential of 53.277 in 0.3 % BSA. No significant ($P>0.05$) differences in mean of ranks of intensity of motility were observed in comparisons involving the following extenders: positive control *vs*. 1 % BSA, positive control *vs*. 5 % BSA, positive control *vs*. 10 % BSA, positive control *vs*. 15 % BSA, negative control *vs*. 0.3 % BSA, negative control *vs*. 1 % BSA, negative control *vs*. 5 % BSA, negative control *vs*. 10 % BSA, 0.3 % BSA *vs*. 1 % BSA, 0.3 % BSA *vs*. 5 % BSA, 0.3 % BSA *vs*. 10 % BSA, 1 % BSA *vs*. 5 % BSA, 1 % BSA *vs*. 10 % BSA, 1 % BSA *vs*. 15 % BSA, 5 % BSA *vs*. 10 % BSA, 5 % BSA *vs*. 15 % BSA, and 10 % BSA *vs*. 15 % BSA.

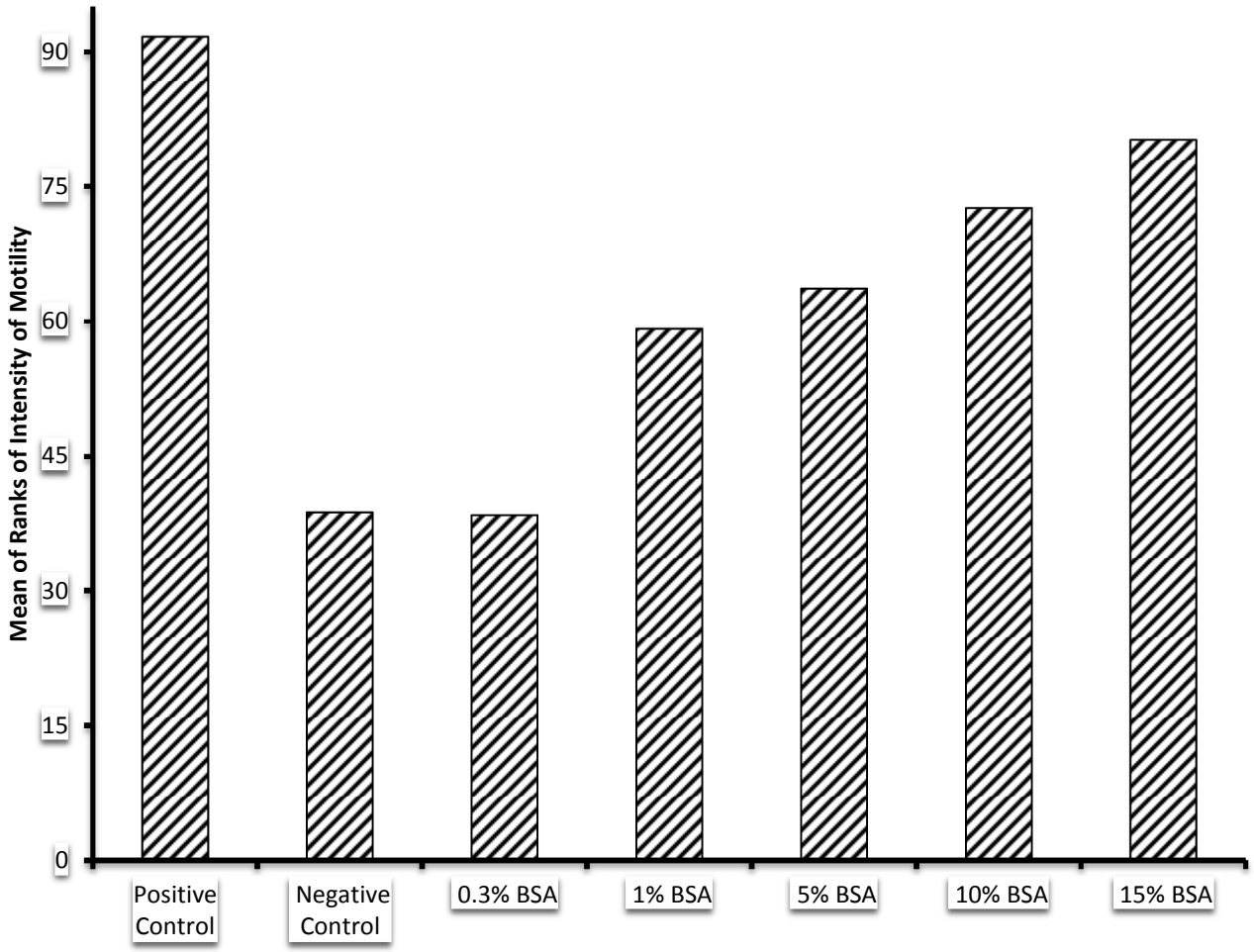

Figure 4.9 Intensity of Motility After Six-Hour Incubation as Affected by Extender

4.1.7 Effect of Individual Ram on Total Motility of Epididymal Spermatozoa

Percent total motility of epididymal spermatozoa as affected by individual ram is shown in Table 4.1. Mean percent total motility (13 ± 3 %) in Ram 1 increased significantly ($P<0.05$) by differentials of 52, 64, 62, 63, 64, 71, 70, 68, 66, 63, 48, 66, 50, 51, 56, 64, and 56 % in Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 9, Ram 10, Ram 11, Ram 12, Ram 13, Ram 14, Ram 15, Ram 16, Ram 17 and Ram 18, respectively. However, there was a significant ($P<0.05$) decrease in mean percent total motility (84 ± 3 %) of epididymal spermatozoa in Ram 7 by differentials of 23, 21, 20, 15 and 15 % in Ram 12, Ram 14, Ram 15, Ram 16, and Ram 18, respectively. Total motility (65 ± 3 %) of epididymal spermatozoa in Ram 2 increased significantly ($P<0.05$) by differences of 19, 18 and 16 % in corresponding values across Ram 7, Ram 8 and Ram 9, respectively. There were no significant ($P>0.05$) differences in percent total motility of epididymal spermatozoa among Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 10, Ram 11, Ram 13, Ram 16, Ram 17 and Ram 18. Similarly, there were no significant ($P>0.05$) differences in percent total motility of epididymal spermatozoa among Ram 2, Ram 12, Ram 14 and Ram 15.

Table 4.1: Percent Total Motility of Epididymal Spermatozoa as Affected by Individual Ram

	Total Motility (%)	
Individual Ram	Mean	SEM
1	13^{g}	3
2	65^{def}	3
3	77^{abcde}	3
4	75^{abcde}	3
5	76^{abcde}	3
6	77^{abcde}	3
7	84^{a}	3
8	83^{ab}	3
9	81^{abc}	3
10	79^{abcd}	3
11	76^{abcde}	3
12	61^{f}	3
13	79^{abcd}	3
14	63^{ef}	3
15	64^{ef}	3
16	69^{bcdef}	3
17	77^{abcde}	3
18	69^{cdef}	3

^{abcdef} Different superscripts indicate difference at $P<0.05$. SEM = Standard Error of the Mean.

4.1.8 Effect of Individual Ram on Membrane Viability of Epididymal Spermatozoa

Percent viability of epididymal spermatozoa as affected by individual ram is presented in Table 4.2. No significant ($P>0.05$) difference in percent viability of epididymal spermatozoa exists among Ram 1, Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 9, Ram 10, Ram 12 and Ram 13. Similarly, no significant ($P>0.05$) difference was recorded among Ram 1, Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 10, Ram 11, Ram 12 and Ram 13. However, a significant ($P<0.05$) difference in epididymal spermatozoa viability exists between Ram 9 (61 ± 2 %) and Ram 11 (74 ± 2 %).

Table 4.2: Percent Viability of Epididymal Spermatozoa as Affected by Individual Ram

	Viability (%)	
Individual Ram	Mean	SEM
1	68[ab]	2
2	66[ab]	2
3	67[ab]	2
4	68[ab]	2
5	68[ab]	2
6	65[ab]	2
7	63[ab]	2
8	67[ab]	2
9	61[b]	2
10	66[ab]	2
11	74[a]	2
12	66[ab]	2
13	67[ab]	2

[ab] Different superscripts indicate difference at $P<0.05$. SEM = Standard Error of the Mean.

4.1.9 Effect of Individual Ram on DNA Integrity of Epididymal Spermatozoa

DNA integrity of epididymal spermatozoa as affected by individual ram is highlighted in Table 4.3. Percent intact DNA integrity of epididymal spermatozoa (73 ± 2 %) in Ram 1 decreased significantly ($P<0.05$) by differences of 9, 6, 6, 7 and 7 % in epididymal spermatozoa of Ram 7, Ram 8, Ram 11, Ram 12 and Ram 14, respectively. However, percent intact DNA integrity of epididymal spermatozoa (64 ± 2 %) in Ram 7 increased significantly ($P<0.05$) by differences of 9, 7, 6, 7 and 6 % in epididymal spermatozoa of Ram 1, Ram 4, Ram 5, Ram 13 and Ram 15, respectively. No significant ($P>0.05$) effect on intact epididymal spermatozoa DNA exists among Ram 1, Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 9, Ram 10, Ram 13 and Ram 15. Similarly, no significant ($P>0.05$) effect on intact epididymal spermatozoa DNA was observed among Ram 2, Ram 3, Ram 6, Ram 7, Ram 8, Ram 9, Ram 10, Ram 11, Ram 12 and Ram 14.

Table 4.3: DNA Integrity (Percent Intact DNA) of Epididymal Spermatozoa as Affected by Individual Ram

	Intact DNA (%)	
Individual Ram	Mean	SEM
1	73[a]	2
2	67[abc]	2
3	70[abc]	2
4	71[ab]	2
5	70[ab]	2
6	67[abc]	2
7	64[c]	2
8	67[bc]	2
9	68[abc]	2
10	67[abc]	2
11	67[bc]	2
12	66[bc]	2
13	71[ab]	2
14	66[bc]	2
15	70[ab]	2

[abc] Different superscripts indicate difference at $P<0.05$. SEM = Standard Error of the Mean.

4.1.10 Multiple Comparison of Intensity of Epididymal Sperm Motility between Individual Rams before 6 h Incubation

Multiple comparison of intensity of epididymal sperm motility between individual rams before 6 h incubation is shown in Table 4.4. The Intensity of epididymal sperm motility before 6 h incubation (mean of ranks = 6.786) in Ram 1 increased significantly by differences of 67.643, 75.214, 75.214, 67.643, 65.786, 75.214, 75.214, 58.214, 64.357, 57.429 and 65.786 in Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 10, Ram 11, Ram 12, Ram 13, Ram 14 and Ram 17, respectively. All other comparisons were not significantly ($P>0.05$) different.

Table 4.4: Multiple Comparison of Intensity of Epididymal Sperm Motility between Individual Rams before 6 h Incubation

Comparison	Mean Rank Difference	P-Value
Ram 1 *vs*. Ram 4	-67.643	P<0.01
Ram 1 *vs*. Ram 5	-75.214	P<0.001
Ram 1 *vs*. Ram 6	-75.214	P<0.001
Ram 1 *vs*. Ram 7	-67.643	P<0.01
Ram 1 *vs*. Ram 8	-65.786	P<0.01
Ram 1 *vs*. Ram 10	-75.214	P<0.001
Ram 1 *vs*. Ram 11	-75.214	P<0.001
Ram 1 *vs*. Ram 12	-58.214	P<0.05
Ram 1 *vs*. Ram 13	-64.357	P<0.01
Ram 1 *vs*. Ram 14	-57.429	P<0.05
Ram 1 *vs*. Ram 17	-65.786	P<0.01

All other comparisons not reflected in the table are not significantly (P>0.05) different.

4.1.11 Multiple Comparison of Intensity of Epididymal Sperm Motility between Individual Rams after 6 h Incubation

Multiple comparison of intensity of epididymal sperm motility between individual rams after 6 h incubation is presented in Table 4.5. Intensity of epididymal sperm motility after 6 h incubation (mean of ranks = 14.571) in Ram 1 increased significantly by differences of 69.500, 77.429, 90.929, 75.857 and 70.500 in Ram 3, Ram 4, Ram 5, Ram 10 and Ram 13, respectively. However, intensity of epididymal sperm motility after 6 h incubation (mean of ranks = 105.500) in Ram 5 decreased significantly ($P<0.05$) by a difference of 68.071 in Ram 16.

Table 4.5: Multiple Comparison of Intensity of Epididymal Sperm Motility between Individual Rams after 6 h Incubation

Comparison	Mean Rank Difference	P-Value
Ram 1 *vs*. Ram 3	-69.500	P<0.05
Ram 1 *vs*. Ram 4	-77.429	P<0.01
Ram 1 *vs*. Ram 5	-90.929	P<0.001
Ram 1 *vs*. Ram 10	-75.857	P<0.01
Ram1 *vs*. Ram 13	-70.500	P<0.05
Ram 5 *vs*. Ram 16	68.071	P<0.05

All other comparisons not reflected in the table are not significantly (P>0.05) different.

4.1.12 Interactive Effect of Incubation and Individual Ram on Total Motility of Epididymal Spermatozoa

The interactive effect of incubation and individual ram on percent total motility of epididymal spermatozoa is depicted in Table 4.6. Total epididymal spermatozoa motility in Ram 1 before 6 h incubation was significantly ($P<0.05$) different when compared with corresponding total motility values across all rams. Total motility before 6 h incubation was not significantly ($P>0.05$) different among Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 9, Ram 10, Ram 11, Ram 13, Ram 14, Ram 15, Ram 16, Ram 17 and Ram 18. Similarly, total motility before 6 h incubation was not statistically ($P>0.05$) significant among Ram 2, Ram 3, Ram 4, Ram 6, Ram 9, Ram 10, Ram 12, Ram 13, Ram 14, Ram 15, Ram 16, Ram 17 and Ram 18. However, total epididymal sperm motility before 6 h incubation in Ram 12 increased significantly ($P<0.05$) by differences of 22, 24, 24 and 23 % in Ram 5, Ram 7, Ram 8 and Ram 11, respectively. Total motility in Ram 1 after 6 h incubation was significantly ($P<0.05$) different when compared with corresponding values across all rams. Epididymal sperm total motility after 6 h incubation was not significantly ($P>0.05$) different among Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 8, Ram 9, Ram 10, Ram 11, Ram 12, Ram 13, Ram 16, Ram 17 and Ram 18. Also, epididymal sperm total motility after 6 h incubation was not significantly ($P>0.05$) different among Ram 3, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 9, Ram 10, Ram 11, Ram 12, Ram 13, Ram 16, Ram 17 and Ram 18. Similarly, no significant ($P>0.05$) difference in epididymal sperm total motility after 6 h incubation exists among Ram 2, Ram 4, Ram 5, Ram 6, Ram 10, Ram 11, Ram 12, Ram 14, Ram 15, Ram 16, Ram 17 and Ram 18. However, epididymal sperm total motility after 6 h incubation in Ram 7 decreased

significantly (P<0.05) by differentials of 28 and 25 % in Ram 14 and Ram 15, respectively.

Table 4.6: Interactive Effect of Incubation and Individual Ram on Percent Total Motility of Epididymal Spermatozoa

		Total Motility (%)	
Incubation	Individual Ram	Mean	SEM
Before 6 h	1	20^{f}	4
	2	69^{abcde}	2
	3	75^{abcde}	6
	4	76^{abcde}	2
	5	82^{ab}	2
	6	77^{abcd}	2
	7	84^{a}	1
	8	84^{a}	2
	9	81^{abc}	2
	10	80^{abcd}	2
	11	83^{a}	2
	12	60^{cde}	9
	13	79^{abcd}	5
	14	72^{abcde}	3
	15	69^{abcde}	6
	16	70^{abcde}	6
	17	78^{abcd}	8
	18	67^{abcde}	5
After 6 h	1	5^{f}	1
	2	60^{bcde}	6
	3	79^{abcd}	2
	4	74^{abcde}	1
	5	70^{abcde}	2
	6	77^{abcde}	3
	7	83^{a}	1
	8	82^{ab}	2
	9	82^{ab}	3
	10	77^{abcde}	4
	11	70^{abcde}	4
	12	62^{abcde}	4
	13	78^{abcd}	2
	14	55^{e}	6
	15	58^{de}	3
	16	69^{abcde}	7
	17	75^{abcde}	5
	18	71^{abcde}	6

^{abcde} Different superscripts indicate difference at P<0.05. SEM = Standard Error of the Mean.

4.1.14 Interactive Effect of Incubation and Individual Ram on Membrane Viability of Epididymal Spermatozoa

The interactive effect of incubation and individual ram on percent viability of epididymal spermatozoa is depicted in Table 4.7. No statistically ($P>0.05$) significant differences were noted in percent viability of epididymal sperm before 6 h incubation among all rams. Similarly, percent viability of epididymal spermatozoa after 6 h incubation was not significantly ($P>0.05$) different among Ram 1, Ram 2, Ram 3, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 10, Ram 11, Ram 12 and Ram 13. Also, epididymal sperm viability after 6 h incubation was not significantly ($P>0.05$) different among Ram 2, Ram 3, Ram 4, Ram 4, Ram 5, Ram 6, Ram 7, Ram 8, Ram 9, Ram 10, Ram 12 and Ram 13. However, percent viability of epididymal sperm after 6 h incubation in Ram 1 was significantly ($P<0.05$) higher than its corresponding value in Ram 9 by a difference of 18 %. On the other hand, percent viability of epididymal sperm after 6 h incubation in Ram 9 was significantly ($P<0.05$) lower than its corresponding value in Ram 11 by a difference of 21 %.

Table 4.7: Interactive Effect of Incubation and Individual Ram on Percent Viability of Epididymal Spermatozoa

		Viability (%)	
Incubation	Individual Ram	Mean	SEM
Before 6 h	1	62[ab]	4
	2	69[ab]	4
	3	69[ab]	3
	4	67[ab]	3
	5	75[a]	5
	6	70[ab]	4
	7	64[ab]	5
	8	66[ab]	4
	9	66[ab]	3
	10	73[ab]	5
	11	72[ab]	5
	12	64[ab]	7
	13	68[ab]	4
After 6 h	1	74[a]	3
	2	62[ab]	5
	3	65[ab]	7
	4	70[ab]	3
	5	62[ab]	5
	6	60[ab]	3
	7	63[ab]	3
	8	67[ab]	4
	9	56[b]	5
	10	60[ab]	2
	11	77[a]	7
	12	68[ab]	6
	13	67[ab]	4

[ab] Different superscripts indicate difference at $P<0.05$. SEM = Standard Error of the Mean.

4.2 DISCUSSION

Freezing-free preservation is a potential alternative to the conventional method of spermatozoa preservation (Nang *et al*., 2011). Epididymal spermatozoa have now become an alternative for the ejaculated semen which was popularly preserved to keep the genetic traits of superior male animals (Foote, 2000). Perhaps because of its easier access and availability at the local slaughter house makes it possible to be harnessed and processed for assisted reproductive biotechnology.

Epididymal spermatozoa recovered from the epididymides which have been preserved at room temperature or 5°C has been reported in bull (Martins *et al*., 2009), boar (Kikuchi *et al*., 1998), stallion (Muradás *et al*., 2006), cat (Tittarelli *et al*., 2006), and dog (Toyonaga *et al*., 2011). The beneficial effect of refrigeration on various parameters of sperm quality, especially motility, may be due to the reduced metabolic rate of sperm cells when it is preserved at 5 ^{o}C (Salamon and Maxwell, 2000). This in agreement with results of the current work where ram cauda epididymal spermatozoa preserved at 5 ^{o}C showed significant acceptable changes in spermatozoa quality parameters.

Egg yolk is generally accepted to be an effective agent in semen diluents for protection of spermatozoa against cold shock and lipid phase transition effects (Drobnis *et al*., 1993; Matsuoka *et al*., 2006). Despite this role of egg yolk, it exhibits variability in terms of sources and lack consistency in semen diluents due to nutritional and environmental differences of the chickens that produced the eggs. Moreover, it is an important source of pathogens such as viruses and bacteria (Bousseau *et al*., 1998). Therefore, it is imperative to develop a synthetic extender that is devoid of egg yolk and has similar or better functions than the conventional

semen extender (Matsuoka *et al*., 2006). Bovine serum albumin was identified as one of the suitable compounds that will overcome in the spermatozoon structural, biochemical and functional changes caused by cold shock and provide it with sources of energy and effective protection (Osman *et al*., 2012). Bovine Serum Albumin has also been used as a substitute for egg yolk in the preservation of rainbow trout and turkey spermatozoa (Bakst and Cecil 1992; Cabrita *et al*., 2001). In the current study, BSA-based extenders were similar or superior to the yolk-based positive control in terms of quality parameters. This implies that BSA could replace egg yolk without detrimental effects to quality parameters of ram epididymal spermatozoa.

The protective properties of BSA on sperm plasma membrane were also reported in dog (Risopatron *et al*., 2002), mouse (Van Thuan *et al*., 2005) and bull (Nang *et al*., 2011). It is possible that BSA increased the stability of membrane structure (Blank *et al*., 1976) or acted as an antioxidant to protect spermatozoa from destruction by free radicals produced as a result of oxidative stress during preservation (Uysal and Bucak, 2007). Development of Intra-Cytoplasmic Sperm Injection-derived embryos improved when mouse spermatozoa were preserved in medium supplemented with BSA (Li *et al*., 2011). Van Thuan *et al*. (2005) have successfully produced healthy rat offspring using spermatozoa preserved in medium containing BSA, indicating that BSA exerts protective effect on spermatozoa DNA. This effect might explain the extended survivability of ram cauda epididymal spermatozoa preserved at refrigeration temperature as revealed in the present study. Moreover, the similarity in terms of DNA integrity between spermatozoa in the yolk-based positive control and other BSA-based extenders could be as a result of the DNA protective effect of BSA.

Osman *et al*. (2012) speculated that at lower concentration, BSA was not efficient in providing enough energy and antioxidant protection to the stored spermatozoa. In addition, Fukui *et al*. (2007) reported that semen extenders containing BSA without egg yolk result in an acceptable fertility which is similar to that achieved with an extender containing egg-yolk. This implies that supplementation of an appropriate amount of BSA to the extender will profoundly help in the use of ram cauda epididymal spermatozoa preserved above freezing point in artificial insemination programmes. However, the major drawback of freezing-free preservation is the short-term preservation period.

Long term cold storage of spermatozoa may lead to a reduction in their motility and damage to the integrity of their membranes and morphology (Jarosz *et al*. , 2016). Some researchers suggested that these changes might be attributed to the accumulation of toxic products of metabolism, especially reactive oxygen species (ROS) (Santos *et al*., 2008). Oxidative stress originating from the generation of ROS causes structural damage to cellular components. Irrespective of the extender used, conditions, temperature of storage, and extension rate, the quality of spermatozoa deteriorates as the duration of storage increases (Salamon and Maxwell, 2000). Findings from this work showed significant reduction in percent total motility, percent viability and intensity of motility after 6 h incubation. Moreover, the reduction in spermatozoa quality parameters after 6 h incubation might be due to the death of some sperm cells which in turn cause inhibitory actions against the remaining cells with an overall effect on quality parameter. It might also be associated with the delicate and sensitive nature of epididymal spermatozoa compared to their ejaculated counterpart due to the lack of seminal plasma which contains natural antioxidants (El-Harairy *et al*., 2016).

Variation in quality of cauda epididymal sperm cells recovered post-mortem has been ascribed to handling conditions or species differences (Lubbe *et al*., 2000; Bertol *et al*., 2013). However, other studies have recorded that quality of epididymal spermatozoa varied according to climatic conditions and temperature (Lone *et al*., 2011). Moreover, Hafez and Hafez (2000) reported that age of the ram and body condition, season of the year, skill of the technician and the frequency of collection affected the ejaculate volume. The current study showed that variation in percent total motility and percent viability of epididymal spermatozoa was explained by the interaction between incubation and individual rams. Moreover, there were individual differences among rams in terms of percent total motility, percent viability, percent intact DNA and intensity of motility before and after 6 h incubation. Nutritional status of the rams could account for the individual differences that exist in the spermatozoa quality parameters. Also, breed and age could also explain these differences.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 SUMMARY

The purpose of this study was to evaluate the effect of bovine serum albumin, incubation and individual ram on some quality parameters of ram cauda epididymal spermatozoa preserved at refrigeration temperature (5^0C). Quality parameters like percent total motility, percent viability, percent intact DNA integrity and intensity of motility were evaluated. Intact scrotal sample collected from one apparently healthy ram of ≥ 3 years of age was sampled per abattoir visit. Three weekly abattoir visits were made consecutively throughout a duration of six weeks. Intact scrotum was obtained and transported to the laboratory. After dissection, spermatozoa were harvested from the cauda epididymis and evaluated for quality parameters (total motility, intensity of motility, DNA integrity and viability) before and after six-hour of incubation. Findings from this work showed that extender supplemented with bovine serum albumin had better performance in preserving quality of refrigerated ram cauda epididymal spermatozoa compared to extender devoid of BSA. Therefore, BSA is a promising replacement for egg yolk in the preservation of ram epididymal spermatozoa.

5.2 CONCLUSION

Percent total motility, percent viability, percent intact DNA integrity and intensity of motility of ram epididymal spermatozoa decreased after 6-hour incubation. Yolk-citrate extender had similar performance with 1, 5, 10 and 15 %

BSA-based extenders in terms of preservation of percent total motility and percent intact DNA of ram epididymal spermatozoa. Individual differences exist among rams in terms of percent total motility, percent viability, percent intact DNA integrity and intensity of motility of ram epididymal spermatozoa before and after 6 h incubation at 5 ^{o}C. Variations in percent total motility and percent viability of epididymal spermatozoa were explained by the interaction between incubation and individual rams.

5.3 RECOMMENDATIONS

The following recommendations were made:

1. BSA (1 to 15%) should be used as a replacement for egg- yolk in the dilution of ram cauda epididymal spermatozoa.
2. Assess lipid peroxidation of the sperm cells at the various levels of BSA incubation.
3. Acrosomal integrity assessment at various levels of BSA incubation.
4. Reactive oxygen species (ROS) assessment.
5. Further studies should be carried out on prolonging the incubation durations from 6 hours to 9 hours or 12 hours.
6. The *in vitro* and *in vivo* fertility of ram cauda epididymal spermatozoa extended in BSA-based diluents should be assessed in future studies.

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