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Effect of storage time on the quality of cauda epididymal spermatozoa of West African dwarf (WAD) rams

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ABSTRACT

The objectives of the study were to develop a protocol for preserving Western African Dwarf (WAD) ram cauda epididymal semen that could be applied in preserving and storing the semen of endangered species/genetically valuable animals in case of death and to study the morphometric characteristic of epididymides after refrigerated storage. Thirty testes-epididymides were collected immediately after slaughter from mature WAD rams and transported in ice chest at (4.5–6 °C) to the laboratory. The samples were either processed immediately or stored in a refrigerator at 5 °C for 6, 12, 24, 48 h. The results indicate that the semen motility decreased ($P < 0.05$) when compared to the control [87.5 ± 2.1% (0 h), to 85.0 ± 1.8% (6 h), 73.3 ± 3.6% (12 h), 53.3 ± 2.5% (24 h) and 50.0 ± 2.9% (48 h)]. The sperm concentration also decreased ($P < 0.05$) as duration of storage increased (0 h) 157.5 ± 8.2, (6 h) 152.3 ± 5.8, (12 h) 125.3 ± 4.4, (24 h) 106.2 ± 2.9, (48 h) 98.5 ± 3.5. Semen viability decreased as duration of storage increased from the 0 h to 48 h [($P < 0.05$; 84.0 ± 1.4%, 82.8 ± 2.2%, 77.3 ± 1.7%, 69.8 ± 1.5%, and 66.5 ± 1.2%, respectively]. Furthermore, there was a decrease ($P < 0.05$) in percentage of intact acrosome as duration of storage increased [(0 h) 90.7 ± 1.0%; (6 h) 89.3 ± 2.0%; (12 h) 85.5 ± 1.6%; (24 h) 70.0 ± 2.4%; and (48 h) 73.3 ± 2.1%]. The results from this study indicate that epididymal semen of WAD rams recovered and preserved at 5 °C for 48 h may be used for artificial insemination.

1. Introduction

In Nigeria, small ruminant populations total about 22.1 million sheep (Afolayan et al., 2001). The West African Dwarf (WAD) breed is a small-bodied, compact breed which has an all-white, black, brown, spotted black or brown on a white coat colour. The breed is considered to be trypanotolerant (resistant to trypanosomiasis; Adu and Ngere, 1979).

Preservation of cauda epididymal spermatozoa is an important technique to conserve biodiversity. Because the number of endangered species is increasing, it is therefore, important to develop an effective protocol for semen from the WAD rams to be harvested after death and preserved with acceptable quality. It, however, is very important to determine the maximum number of hours for which the testicles can be stored at 5 °C while maintaining acceptable quality, because at times the death of an animal is unpredictable and the processing laboratory might not be close to the location of the animal body. Semen recovered from cauda epididymides can be used in the conservation of animal genetic material and for conducting reproductive biotechnology techniques (Abu et al., 2016). Any plan of action for *ex situ* conservation of animal genetic material of valuable and or endangered male animals,

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however, requires protocols for the recovery of viable spermatozoa from the cauda epididymides of slaughtered/dead animals. This becomes necessary when mating or the use of ejaculated semen is not possible due to difficulty of handling intractable animals or sudden death of animals with great genetic value (Foote, 2002; Kaabi et al., 2003; Edeneil et al., 2015).

Genetic improvement of farm animals may rely on the use of limited number of males either for natural mating or in artificial insemination programmes Lone et al. (2011); Ibrahim et al. (2012); Abu et al. (2016) however, have reported that quality of epididymal spermatozoa varied as a result of ambient temperatures and climactic conditions. Furthermore, the method used to recover cauda epididymal spermatozoa reserves post-mortem does not affect the quality of spermatozoa (Abu et al., 2016; Ringleb et al., 2011). Acceptable progressive motility and viability of spermatozoa recovered post-mortem from cauda epididymides that was maintained at 5 °C for 48 h was reported for boars (Kikuchi et al., 1998), bulls (Martins et al., 2009), and stallions (Muradás et al., 2006). In Nigeria, there has been very little research regarding spermatozoa management after an animal has died (Red Sokoto goat bucks (Abu et al., 2016); Nigerian breed of dogs (Chima et al., 2017).

The objectives of the present study, therefore, were (i) to develop a protocol for preserving cauda epididymal spermatozoa of WAD rams that could be applied in preserving and storing the spermatozoa of endangered species/genetically valuable animals and (ii) to study the morphometric characteristics of epididymides of WAD rams stored for different durations of time at 5 °C.

2. Materials and methods

2.1. Study location

The study was conducted in the dry season between February and April in the Makurdi Metropolis of the Benue State of Nigeria, the semen was processed at the Department of Theriogenology laboratory, Federal University of Agriculture, Makurdi. Benue State Nigeria. Makurdi is situated in the southern guinea savannah and located at Latitude 7° 14′ North and Longitude 8° 21′ East, the area has a warm temperatures ranging from 24 to 40 °C. The relatively greater temperatures occur between March and May. The average rainfall is between 508 and 1016 mm annually. Two annual seasons are experienced which are: the rainy season (May-October) and the dry season (November-April; Timi and Tor, 2006).

2.2. Sample collection

Fresh testes-epididymides contained in scrotal sacs were collected from mature WAD rams immediately after slaughter at a Metropolitan Abattoir (Wurukum) in Makurdi, Benue state, Nigeria. Samples were individually packaged in polythene bags and transported in an ice chest at (4.5–6 °C) to the laboratory for further processing. On arrival the samples were divided into six per treatment time ($n = 6$) the 0 h (control were processed at room temperature immediately on arrival to the laboratory) and the remaining samples were stored at 5 °C in refrigerator to be processed at 6, 12, 24, and 48 h post-slaughter, respectively.

2.3. Experimental procedure

The epididymides along with testes were thawed at room temperature (35 ± 2 °C) for 15 min and then immersed into a water bath at 37 °C for 5 min until the outer surface of the testes as well as epididymides were in the thawed state. Testicles were dissected from the tunica vaginalis and other extraneous tissues during the period when tissues were thawing. A scalpel blade was used to make an incision from the dorsomedial aspect of the testis. The skin of the scrotum was reflected laterally, and the subcutaneous tissue and scrotal fascia were incised to expose the tunica vaginalis. The tunica albuginea was carefully removed from the testis. The epididymis was then carefully separated from the testes using the scalpel blade and thumb forceps. The testes and epididymides were excised, separated and weighed on a sensitive balance scale. Spermatozoa were collected from the cauda epididymides at room temperature by the incision method (Abu et al., 2016). Several longitudinal incisions were made at the terminal end of the cauda epididymides to enable spermatozoa swim out into pre-warmed (37 °C) 3 ml, 2.9% sodium citrate buffer in a petri dish. There was 1 ml of the solution used to rinse the cauda epididymis.

2.4. Epididymal morphometry

Epididymal lengths were evaluated using a piece of thread and the lengths were obtained using a ruler with the procedures being utilized that have been previously reported (Osinowo et al., 1981). All weighing of samples was conducted using a sensitive balance in the laboratory.

2.5. Determination of spermatozoa progressive motility

Spermatozoa total progressive motility of different storage groups including control (0 h group) were evaluated by placing a drop (10 µl) of semen sample on a pre-warmed, grease-free slide. A cover slip was placed over the drop and there was examination of the sample using the high-power objective (×40 magnification) of a microscope, and the percentage of progressive motility was determined using the method recommended by Zemjanis (1970).

2.6. Determination of semen concentration

Sperm concentration of each sample was determined using the improved Neubauer haemocytometer after dilution in 0.05% formol-saline (Rekwot et al., 1987; Eljarah et al., 2013). A 1:20 dilution of the sperm sample in 0.05% formol saline was used for this determination. A specific amount (10 µl) of each of the semen sample was placed on both sides of the haemocytometer. The number of sperm cells in five of the large central squares (made up of 16 smaller squares) were counted on both chambers and the average was calculated. Sperm concentration of the sample was obtained using the formula; number of spermatozoa × multiplication factor (50,000) × dilution factor (20) (Rekwot et al., 1987). The results were expressed as concentration/ml.

2.7. Determination of spermatozoa viability

Viability of sperm cells was determined by placing a drop (10 µl) of semen sample on a clean, grease-free slide and mixed with a single drop of eosin-nigrosin stain. The spermatozoa were allowed to interact with the stain for at least 2 min and then a smear was prepared. The prepared smear was air-dried and examined using an oil immersion objective (×100 magnifications). Spermatozoa that stained either partially or completely were considered to be dead, and spermatozoa that appeared colourless were considered to be viable. Spermatozoa (n = 200) were randomly examined in different fields and the percentage of live sperm cells was determined. The mean results were expressed as percentage viable spermatozoa (Blom, 1973).

2.8. Determination of acrosome integrity

Acrosome status was determined by adding (10 µl) a drop of the sample on a clean slide and making a smear. The smear was air-dried and kept in Hancock's fixative for 15–20 min in a coupling jar. The slides were washed using slow running tap water for another 15–20 min and were subsequently rinsed with distilled water. The slides were stored in a coupling jar containing Giemsa working solution overnight. The next day, the stained slides were rinsed using slow-running tap water, air-dried, and observed using an oil immersion objective (×100 magnification). Spermatozoa (n = 200) were randomly examined in different fields and the percentage of spermatozoa with an intact acrosome was determined. The mean results were expressed as percentage intact acrosomes (Watson, 1975).

2.9. Determination of spermatozoa morphological abnormality

Spermatozoa morphological abnormality was determined by viewing the Gyms stained slides. Spermatozoa (n = 200) were examined in different fields and the percentage of spermatozoa with abnormal morphology was determined. The mean results were expressed as percentage of spermatozoa with abnormal morphology (Watson, 1975).

2.10. Statistical analysis

The percentages of the raw data were converted to arcsin values. The Gaussian assumption for all the data was assessed utilising a normality test (Kolmogorov-Smirnov) before subjecting the data to a one-way ANOVA. For progressive motility the probability was greater than 0.05 for all groups except at 12 h of storage, therefore, these data had a normal distribution and an ANOVA was directly applied. Normality results for intact acrosomes were less than 0.05 meaning that the data were not normally distributed and, therefore, the data were converted before subjecting data to a one-way ANOVA. Probability values for normality results related to spermatozoa viability were all less than 0.05 meaning that these data were not normally distributed and, therefore, data were converted before these data were analysed using a one-way ANOVA. Probability values for the normality test for spermatozoa morphology abnormalities were greater than 0.05 for all groups and, therefore, these data were normally distributed and a one-way ANOVA was utilised for data analyses. Also raw data were subjected to the leven test using R Core Team, 2018. R: A language and environment for statistical computing (R Foundation for statistical computing Vienna, Austria). The probability values for results for sperm progressive motility, intact acrosome, viability, concentration and abnormal morphology all were associated with a P-value > 0.05 indicating that variances were homogenous before subjecting these data to a one-way ANOVA.

Data were expressed as mean ± standard error of the mean using the Graph Pad Prism version 4.0 for windows. The differences between means were analysed using the Tukey's multiple comparisons test. The level of significance was set at 5%.

3. Results

3.1. Epididymal biometry

The mean values for epididymal lengths, and weights for each storage duration (0, 6, 12, 24, and 48 h) for both testes are presented in Table 1. There were no differences ($P > 0.05$) in weight and length of the right and left epididymis when there was 0, 6, 12, 24 and 48 h of storage, respectively.

Table 1
Mean (± SEM) epididymal biometry of WAD rams recovered post slaughter, processed immediately without storage (0 h) and after storage at 5 °C for 6, 12, 24, and 48 h.

Variables		Storage	Time (hours)		
	0	6	12	24 S	48
Right epididymal weight (g)	9.20 ± 0.65	10.65 ± 0.58a	9.46 ± 0.79	10.93 ± 1.13	6.25 ± 0.88
Left epididymal weight (g)	8.96 ± 0.56	10.71 ± 0.50	9.93 ± 0.94	11.75 ± 2.24	6.08 ± 0.80
Right epididymal length (cm)	13.81 ± 0.59	13.18 ± 0.32	13.53 ± 0.71	13.26 ± 0.51	12.08 ± 0.78
Left epididymal length (cm)	13.98 ± 0.90	13.91 ± 0.30	14.08 ± 0.80	13.43 ± 0.45	12.50 ± 0.64

Means without superscripts in the same column of paired organs are not different ($P < 0.05$); SEM = Standard Error of Mean.

3.2. Epididymal spermatozoa evaluations after recovery

3.2.1. Spermatozoa motility

Mean percentage spermatozoa motility of each storage time is presented in Table 2. Semen motility decreased ($P < 0.05$) as duration of storage from 12, 24 and 48 h increased. There were no differences ($P > 0.05$) in spermatozoa motility between 0 and 6 h, and also between 24 and 48 h of storage.

3.2.2. Spermatozoa concentration

There were no differences ($P > 0.05$) in the mean spermatozoa concentration between the 0 h and 6 h of storage. There, however, were differences ($P < 0.05$) between the 0 h and 12 h, between 24 and 48 h. There were no significant differences ($P > 0.05$) between 12 and 24 h of storage time, but there were differences ($P < 0.05$) between 12 and 48 h of storage. There were also no significant difference ($P > 0.05$) between 24 and 48 h storage time for spermatozoa concentrations (Table 2).

3.2.3. Spermatozoa viability

There were no differences ($P > 0.05$) in the mean percentage spermatozoa viability between 0 and 6 h, 6 and 12 h and 24 and 48 h of storage. At 48 h of storage, spermatozoa viability was less ($P < 0.05$) when compared with that at the 0 h (Table 2).

3.2.4. Acrosome integrity

There were no differences ($P > 0.05$) in the mean percentage acrosome integrity between 0 and 6 h nor between 6 and 12 h, but there was a difference ($P < 0.05$) between 24 and 48 h for acrosome integrity (Table 2).

3.2.5. Spermatozoa abnormalities

There was an increase in spermatozoa abnormalities as duration of storage increased (Table 2). The mean percentage of spermatozoa abnormalities at the 48 h storage time was greater ($P < 0.05$) than the 0 h time. There, however, were no differences ($P > 0.05$) between 0 and 6 h, 12 and 24 h and 24 and 48 h of storage.

4. Discussion

There was an investigation of the effect of storage time on epididymal biometry variables and quality of spermatozoa reserves in the cauda epididymis when abattoir-derived samples were processed immediately (0 h) or stored at 5 °C for 6, 12, 24, and 48 h, respectively. The decrease in spermatozoa motility, concentration, viability, and intact acrosomes recovered post-slaughter and stored for 6, 12, 24 and 48 h at 5 °C observed in this study have been attributed to accumulation of toxic products of metabolism primarily oxygen reserves. As a result, there is lipid peroxidation of the spermatozoa membrane as has been reported by Kishikawa et al. (1999) in mice, Kikuchi et al. (1998) in boars, Foote (2002) in bulls, Weiss et al. (2008) in stallions and Tajik and Hassan-NejadLamsoo (2008) in camels. Furthermore, there have been similar findings in studies of Nigerian breeds of dogs (Chima et al.,

Table 2
Mean (± SEM) epididymal semen variables of WAD rams recovered post-slaughter, processed immediately after recovery without storage (0 h) and after storage at 5 °C for 6, 12, 24 and 48 h.

Variables		Storage	Time (hour)		
	0	6	12	24	48
Motility (%)	87.5 ± 2.1^{a}	85.0 ± 1.8^{a}	73.3 ± 3.6^{b}	53.3 ± 2.5^{c}	50.0 ± 2.9^{c}
Conc.(million)	157.5 ± 8.2^{a}	152.3 ± 5.8^{ab}	125.3 ± 4.4^{c}	106.2 ± 2.9^{d}	98.5 ± 3.5^{d}
Viability (%)	84.0 ± 1.4^{a}	82.8 ± 2.2^{a}	77.3 ± 1.7^{b}	69.8 ± 1.5^{bc}	66.8 ± 2.0^{c}
Intact acrosome (%)	90.7 ± 1.0^{a}	89.3 ± 2.0^{a}	85.5 ± 1.6^{a}	79.0 ± 2.4^{c}	73.3 ± 2.1^{d}
Abnormal Morphology (%)	9.2 ± 1.9^{a}	11.8 ± 1.2^{a}	21.0 ± 1.5^{b}	26.5 ± 1.2^{bc}	31.8 ± 2.0^{c}

Means with different alphabet superscripts in the same rows are different ($P < 0.05$); SEM = Standard Error of Mean.

2017) and the Red Sokoto goat buck (Abu et al., 2016). Variation in quality of cauda epididymal sperm cells recovered post-mortem have been ascribed to handling conditions or species differences (Lubbe et al., 2000; Bertol et al., 2013; Abu et al., 2016).

Sperm concentration varies not only among animals but also among species of livestock (Bergeron & Manjunath, 2006). The results of the present study indicate epididymal spermatozoa of WAD rams had a low percentage motility after 48 h of storage at 5 ºC compared to the 0 h value. These findings are consistent with those of Abu et al. (2016) where it was reported there was a lesser spermatozoa motility after 48 h of storage of Red Sokoto goat buck epididymides at 4 °C. Goodrowe and Hay (1993) reported similar results for cat epididymides after overnight storage at 5 °C. Mir et al. (2012) reported there was a decreased motility after 48 h of epididymides storage at 4 °C. Kilian et al. (2000), after 108 h of storage at 4 ºC, reported similar results for African buffalo epididymal sperm motility. Strand et al. (2016) reported that there was lesser bull spermatozoa motility as compared with that at the time of epididymides collection when bulls were between 37 and 51 weeks and for bulls between 52 and 115 weeks of age after 48 h of epididymal storage at 5 °C. There were differences ($P < 0.05$) in the mean epididymal spermatozoa concentration between 0 h and 48 h of storage which was also reported by Abu et al. (2016) in a study with Red Sokoto goat bucks, Mir et al. (2012) in Indian indigenous rams, Chima et al. (2017) in a Nigerian breed of dogs, and Hori et al. (2004) in Beagle dogs. The possible reasons for the decrease in spermatozoa concentration as duration of storage increased is that in live animals, the cauda epididymides provides an optimal environment for the immature spermatozoa to mature and acquire motility. Furthermore, results of studies indicate that spermatozoa from cauda epididymides become motile only when spermatozoa come in contact with seminal fluid or media (Amann and Almquist, 1962; Lima et al., 2013), however, these processes do not occur with collection of spermatozoa post-mortem. There are also reports that the handling conditions of testicles recovered post-mortem affect the concentration of spermatozoa (Foote, 2002). The enhanced spermatozoa metabolic rate and degeneration of sperm cells post-mortem can lead to a reduction in sperm concentration. There was a decrease in sperm viability as storage time increased in the present study. This finding is consistent with those from a previous study in dogs of a Nigerian breed (Chima et al., 2017) and Red Sokoto goat bucks (Abu et al., 2016).

The success of assisted reproductive techniques such as artificial insemination (AI) and in *vitro* fertilization (IVF) depends on the use of a sufficient number of motile spermatozoa with an acrosome intact that have the potential of contributing in the fertilization process with the oocyte (Reckova et al., 2015). In the present study, there was a lesser ($P > 0.05$) mean percentage of spermatozoa with an intact acrosome as duration of storage increased. These findings are consistent with previous results of a study with Red Sokoto goats (Abu et al., 2016) and Indian indigenous rams (Mir et al., 2012). The mean percentage spermatozoa morphological abnormalities increased as duration of storage increased in the current study. These results are consistent with the findings of Hori et al. (2004) in Beagle dogs, and by Hoseinzadeh-Sani et al. (2013) in Iranian goats where it was reported there was an increase after 0 h and at 48 h of storage time in spermatozoa tail abnormalities. This could be attributed to the effects of changes in osmotic pressure of the epididymal fluid due to storage at lesser temperatures than occurs *in vivo* (5 ºC).

Ultra structural damage during refrigerated storage is accompanied with biochemical changes, some of which are major losses from spermatozoa including glutamic oxaloacetic transaminase (GOT), lipoproteins and amino acids. Furthermore, with ultrastructural damage to spermatozoa, there is a decrease in phosphatase activity, a decrease in loosely bound cholesterol protein, an increase in sodium and decrease in potassium content, an inactivation of hyluronidase and acrosin enzymes, a loss of prostaglandins, a reduction of ATP and ADP systems, and a decrease in acrosomal proteolytic activity (Salaman and Maxwell, 1995). Denaturization of DNA can also occur, as modifications in the spermatozoa chromatin structure of bulls, boars, cats, humans and rams has been observed (Gillan and Maxwell, 1999). These ultrastructural and biochemical cryogenic changes to spermatozoa could be responsible for a decrease in the functional integrity *in vivo* and fertilization capacity. Although the motility, viability and acrosome integrity of the spermatozoa are within the acceptable limits after 48 h of storage at 5 °C this does not mean that these spermatozoa will have the capacity for fertilisation if utilized for AI. This is because cold storage in ram sperm will induce apoptotic and capacitating-like changes, along with DNA damage that will reduce spermatozoa fertilising capacity.

Results of the present study indicate it is possible to recover the testes-epididymides from WAD rams that have been incapacitated, making them incapable of naturally mating, or with any form of retractable illness or unexpected death. If the spermatozoa cannot be processed immediately, temporary storage or transport of the testicle at 5 °C for as long as 48 h will preserve the cauda epididymal sperm cells so that spermatozoa can subsequently be recovered for use in AI with assisted reproductive technologies. This process will allow for continuation of the propagation of rams with high genetic value. This finding could also be extended to other animal species.

Conflict of interest

The three authors have all contributed in the samples collection, processing and writing of this manuscript. The research was funded by the authors with the assistance from the University. The authors has agreed that the manuscript should be send to the Journal of Animal Reproduction Science. Therefore, there is no conflict of interest between the authors and also the institution.

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