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The characterization of Post-Mortem Sperm of Local Chicken Cocks in Eastern Algeria

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ABSTRACT

The present aimed to investigate for the first time the characteristics and conservation of post-mortem sperm, obtained from epididymis and the vas deferens of 18 pairs of adult local chicken cocks from the east of Algeria (age, 12-24 months, body weight 1.50-2.53 kg). And compare the sperm quality at the epididymis and the vas deferens levels, the efficacy of two post-mortem sperm retrieval techniques, the flushing and float-out methods in the collection of local chicken cock's sperm, and the effects of conservation in situ at different temperatures (2h and 24h at 20°C and after refrigeration at 4°C for 24h). The quality was significantly higher (0.13±0.05ml vs 0.72±0.12ml) in the vas deferens compared to the epididymis, whereas spermatozoa abnormalities and chromatin quality did not differ in both the epididymis and vas deferens. No significant difference was observed between the flushing and float-out methods. Concentration (3.33±1.63 million sperm vs 1.75±0.76 million sperm), initial motility (77.50±6.89% vs 75.83±8.61%), viability (75±10.39% vs 74.67±10.15%) and abnormality (30.33±4.68% vs 30.33±4.68%), only the volume was significantly higher (0.72±0.12ml vs 0.17±0.08ml). However, the effects of conservation in situ, at 25°C and 4°C for 24h, showed a significant difference for viability and motility of the spermatozoa recovered from vas deferens, and no significant difference for abnormality and acrosome integrity. Therefore, it can be concluded that good quality semen samples can be collected from the vas deferens with the flushing method, and semen of Algerian local cocks can be preserved at 20°C for 24h.

Key words: Conservation, Eastern Algeria, Epididymis, Local cocks, Post-mortem, Vas deferens

INTRODUCTION

Actually, a plethora of research focused on the conservation of genetic resources and biodiversity (Blesbois, 2007). The importance of granting poultry genetic resources from unexpected accidents or disease outbreaks cannot be overemphasized (Sasaki et al., 2010). Local poultry breeds may cause an interesting different option to commercial lines, supplying high-quality products of great interest for local and regional markets (De Marchi et al., 2005). Poultry products, meat and eggs, obtained from native breeds show specific features (De Marchi et al., 2005; Castellini et al., 2006; Zanetti et al., 2011) which distinguish them from standard ones, moreover, breeds can be reared in outdoor free-range systems and even used to reintroduce agricultural activity in marginal rural areas. Traditionally, the creation of banks of semen doses from endangered breeds is performed using the protocols developed for commercial breeds or lines (Blesbois, 2007).

In addition to experimental lines and commercial stocks contributing to the range of chicken genetic diversity, traditional chicken breeds are still highly connected to cultural values, geographical origins and adapted to local environments; however, many of them have undergone a major decrease in their population size, thus increasing erosion of their genetic diversity (Woelders et al., 2006). The loss of valuable genes and rapid decrease in biodiversity as a result of a smaller number of selected breeds used for breeding has resulted in an urgent need to create gene banks and databanks

(Tisdell, 2003). In poultry species, semen cryopreservation is currently the most practical method for long-term storage of genetic material (Blesbois, 2007).

The most frequent method of semen collection in birds is Dorso-abdominal massage (Chelmonska et al., 2008). But it has been proven that this method of harvesting is harmful to sperm due to sperm contamination during urinary retrieval (Blanco et al., 2002). The use of the post-mortem harvest of spermatozoa by the various methods (like Swim-up and mincing), was a way of overcoming the inconveniences of the abdominal massage, which has already been found in Philippine roosters (Salting et al., 2016). Germplasm banks for endangered avian species are still being set up. Gunn et al. (2008) described and compared different techniques for post-mortem sperm collection in wild pukekos (*Porphyrio porphyrio melanotus*), although none of the cells obtained was motile. Some authors, because they did not evaluate the viability of the sperm nor the effectiveness of its extension suggested that the pukeko sperm is not mobile in the vas deferens and becoming active only after ejaculation, or that those recovered are simply dead (Gunn et al., 2008).

Sadly, the avian spermatology is not simple, which is necessary to improve sperm cryopreservation (Villaverde-Morcillo, 2016). However, sperm may also be useful to create germplasm banks of sperm from dead birds by feeding information on the physiological status of the donor used for reproduction (Kumar and Holt, 2014). Semen evaluation prior to its further processing is essential and an important prerequisite. In order to propose the devising methods for the storage of fowl spermatozoa, it is necessary to evaluate the basic quality of sperm (Łukaszewicz et al., 2008). To ensure the better cryopreservation, it is essential to appreciate the mobility, morphology and the metabolism of fresh semen (Blesbois, 2007). This study was conducted to characterize post-mortem sperm of local Algeriancocks collected from the epididymis and the vas deferens, find the suitable methods of extract the sperm and the effects of conservation in situ at different temperatures (2 hours and 24 hours at 20°C and after refrigeration at 4°C for 24 h).

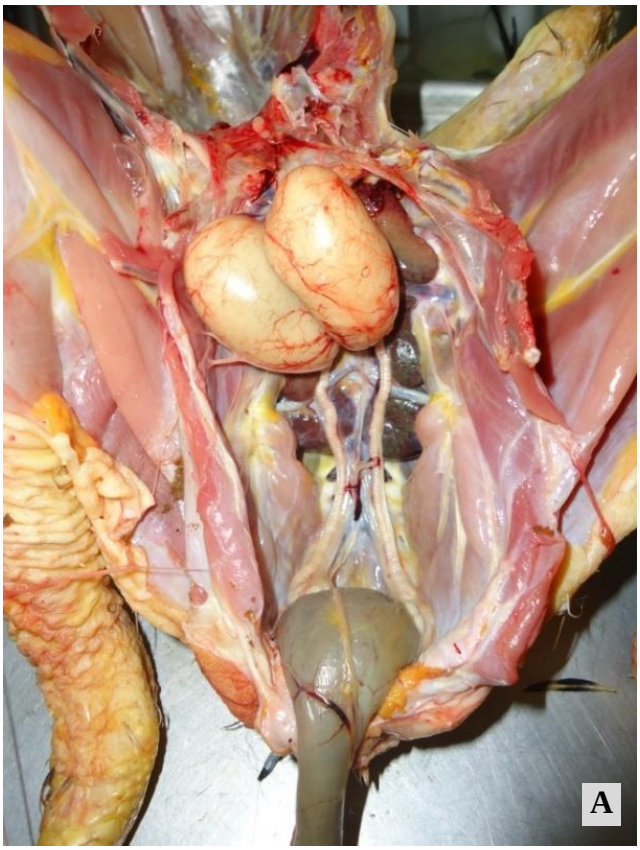
MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the national regulations on animal welfare and institutional animal ethical committee of national center for biotechnology research, Constantine, Algeria.

Semen collection and evaluation

The birds used in present investigation were local cocks from the east of Algeria (El Tarf and Constantine). 12-24 months of age and 1.50- 2.53kg live body weight were used in this experiment. Procedures were performed using sperm obtained post-mortem from the epididymis and the vas deferens of 18 pairs adult cockshumanely euthanized by cervical dislocation. Testes with their corresponding vas deferens were collected within one hour after slaughtering by opening the carcass along the midline. All the viscera were removed and the testes extracted together with the ureter and renal vasculature, thus damages to the vas deferens was avoided. The ureter and renal vasculature were carefully dissected out to minimize blood and urine contamination of the sperm sample (Figure 1). Once the epididymis and vas deferens were isolated from the testes and adjacent tissues, sperm collection was performed either by the flushing or float-out method from the vas deferens (one testis via the flushing method, and the other by the float-out method), and the epididymis employed simply by the mincing method. Following sperm collection, sperm variables were assessed before refrigeration and 24 hours at environment temperature, then after cooling in situ at 4°C for 24 hours.



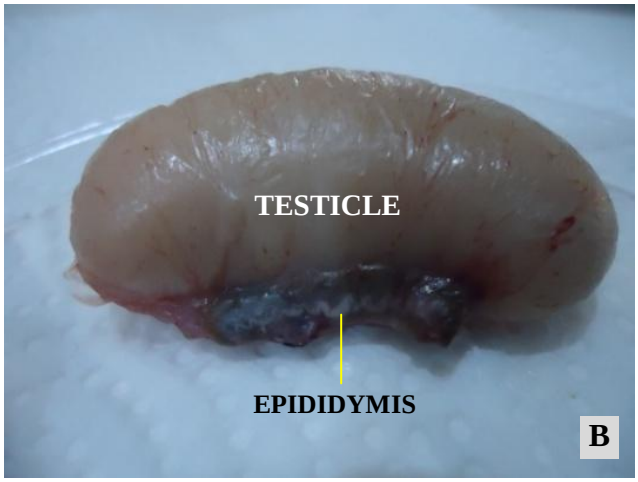


Figure 1. Anatomy of reproductive tract of adult local cocks' in Eastern Algeria(A and B)

Mincing method

The Mincing technique involves isolating the epididymis from the testis, placed in microcentrifuge tubes containing 400µl of Phosphate Buffered Saline (PBS) and were minced by using sterile surgical scissors, and then left to stand for 10-15 minutes. The upper liquid portion was transferred to another microcentrifuge tube (Salting et al., 2016).

Flushing method

Samples were obtained by injecting 1.5 ml of saline solution at 38°C into the proximal extreme of the vas deferens using a 27G needle attached to a 5 ml syringe (Figure 2). The entire volume of the saline solution and the collected sperm were placed in a sterile plastic Petri, from which they were transferred to a polystyrene tube (2 ml). The samples were then incubated at 38°C for 15 min (Villaverde-Morcillo, 2016).

Figure 2. Extraction the sperm from Vas deferens 2hours after slaughtering of local cocks' in Eastern Algeria by flushing method

Float-out method

The vas deferens was cut into 0.5 cm-long pieces which were submerged in 1.5 ml of saline solution at 38°C in a 2 ml tube. These samples were then incubated at 38°C for 15 min (Villaverde-Morcillo, 2016).

Sperm assessment

Semen volume (ml) was measured visually using a graduated collection tube. The total volume retrieved after flushing or float-out was considered as the final sperm volume to calculate sperm concentration. The concentration of spermatozoa (million per millilitre) was determined by using a calibrated spectrophotometer. The wavelength is set at 650 nm. The addition of 3 ml of a 2.9% sodium citrate solution (PH 7.0). A drop of semen (10 µl) was placed on a clean, pre-warmed (41°C) microscope slide using a micropipette. A clean cover slip was placed gently to avoid air bubble formation. Sperm motility was demonstrated by the progressive wavy movement of sperm cells. Briefly, 20 µl aliquot from sperm suspension was stained by 20 µl Eosin–Nigrosine dye. Then, smears were prepared on a warm slide and the stain spread with a second slide. Twenty hundred sperm were counted under phase-contrast at 1000× magnification. Acrosomal integrity was studied using Giemsa staining (Watson, 1975).

Acidic aniline blue staining

Sperm chromatin condensation was disturbed (NCI) when lysine-rich somatic histones were not sufficiently substituted by arginine-rich protamines during spermiogenesis. Histone-containing spermatozoa can be visualized using acidic aniline blue staining solution, which reacts with lysine residues of persisting histones (Shanmugam et al., 2016). The percentage of sperm heads partially or entirely stained dark blue was examined according to the procedures described by Boitrelle (2004).

Statistical analysis

Data were analyzed using SPSS software (version 19). The effect of the level of epididymis or the vas deferens, two sampling techniques (Float-out and flushing methods), and the time of conservation of the gonads (2h, 24h at 25°C and 24h at 4°C) on the sperm quality were compared by the one-way ANOVA test followed by the Student Newman-Keuls multiple comparison test. Mean values were considered significantly ($P<0.05$), data are expressed as values ± Standard Deviation (SD).

RESULTS AND DISCUSSION

Various parameters of spermatozoa collected from the epididymis and the vas deferens

Mean values of volume, initial motility, concentration, viability, abnormality and aniline blue negative spermatozoa (chromatin quality) were presented in table 1. The volume, concentration, motility and viability of spermatozoa were significantly (P<0.05) influenced by the level of sampling. They were significantly higher (P<0.001) in the vas deferens compared to the epididymis. While spermatozoa abnormalities and chromatin quality do not differ in the epididymis and the vas deferens (P>0.5).

Table 1. Comparison of sperm quality at the level of the epididymis and the vas deferens from local cocksin Eastern Algeria

Items	Epididymis	Vas deferens	P
N	12	12	value
Volume (ml)	0,13±0,05	0,72±0,12	***
Concentration (10^6)	250±137,84	5333,33±1861,90	***
Initial motility (%)	65,83±4,92	80,83±7,36	**
Viability (%)	53,17±9,13	86,50±2,59	***
Abnormality (%)	21,17±3,37	20,67±13,43	ns
Aniline blue negative (%)	78,17±4,75	76,17±6,62	ns

Nombre (n), no significant difference (ns)** P<0.01 and *** P<0.001

The results confirmed the findings of Munro (1935 and 1938), which reported that sperm motility increased somewhat in the rudimentary epididymis of birds, but the maximum motility was seen in the vas deferens. Avian spermatozoa are not motile in the testes, although they have been found to be capable of fertilizing eggs when taken from the testes. Because spermatozoa are mature at this stage (Jones and Lin,1993). The role of epididymal region of the rooster reproductive tract is the production of fertile sperm. But testicular effluent is concentrated and surface of sperm cells altered by passing through the excurrent ducts of the epididymis (Clulow and Jones, 1988). These changes presumably revealed base on differences in the function of sperm recovered from the testis and deferent duct (Howarth, 1983). Clulow et al. (1988) reported that before the sperm stored for an extended period in the ductus deferens, these ducts resorb nearly 90% of the testicular plasma outputwhen the sperm traverse this region of the male reproductive tract rapidly. The assessment of the basic quality of sperm is indispensable for the purpose of devising methods for the storage of fowl spermatozoa *in vitro* for long periods of time (Łukaszewicz et al., 2008). Salting et al. (2016) expressed for choosing semen at good quality level, colour must be free of any contamination caused by cloacal products, the volume greater than 0.3 ml, sperm mobility greater than 65%, and sperm concentration greater than 1×10^9 sperm cells /ml. This study addressed the contribution of the epididymis to the attainment of functional maturity of local cocks' spermatozoa. In contrast to their mammalian counterparts, where it is well established that spermatozoa undergo a number of important changes in the epididymis, present study obtained few changes in the functional profile of indigenous cocks spermatozoa at epididymis. These results are in accordance with the fundamental differences in the anatomy and biology of the avian and mammalian reproductive systems (Jones et al., 2003). Accordingly, the vas deferens (leading from the testes to the cloaca) should be the ideal site to harvest mature sperm (Gunn et al., 2008). The results of the study proved that good quality semen samples can be collected from the vas deferens of local cocks with parameters comparable to ejaculated sperm of some strains. Samples collected from the vas deferens can be considered as a better source for future rooster semen studies regarding the development of methods of chicken sperm cryopreservation and, eventually, in cryopreservation per of valuable genetic resources. Because, the basis of sperm can be a suitable indicator in AI practices (Mavi et al., 2017).

Spermatozoa retrieved of the vas deferens by float-out and flushing methods

This study was the first comparing two post-mortem sperm retrieval techniques of cocks' sperm from the vas deferens of Algerian local cocks, retrieved by flushing and float-out methods. Table 2 presents the comparison of motility, viability, concentration and percent abnormality observed in the samples retrieved using flushing method and float-out method. It indicates that the volume of sperm (ml) retrieved by flushing was 0.72±0.12, but with the float–out method, it was 0.17±0.08. The difference was significantly higher (P<0.01), the volume harvested by float-out method was lower than Aseel (0.36 ± 0.08) and Kadaknath (0.30 ± 0.06 ml) breed (Mavi et al., 2017), and of Venda cockerels (0.3ml) (Mphaphathi et al., 2016), obtained by abdominal massage technique.

The concentrations of sperm (billion cells per millilitre) retrieved by flushing and float-out methods found were 3.33±1.63 and 1.75±0.76, respectively. The t-test did not obtain significant (P>0.05) differences between the concentrations of samples retrieved using

flushing and float-out methods. In the present study, the methods of collecting caused low concentration than harvested by abdominal massage technique (6.8×10^9/ml) from Venda chicken (Mphaphathi et al., 2016). Siudzinska and Lukaszewicz (2008) reported an average sperm concentration of 4.7×10^9/ml in White Crested Black Polish cocks and 4.2×10^9/ml in the Black Minorcas breeds. Tuncer et al. (2008) and Obidi et al. (2008) reported sperm concentrations at levels of 2.4×10^9/ml in Gerze cocks and 3.6×10^9/ml in Shikabrown cocks similar to present findings.

The study of Villaverde-Morcillo et al. (2016) demonstrated the interaction between recovery methods and sperm diluents did not exert significant influence on sperm variables. Irrespective of the extender used, significantly more sperm was retrieved by the flushing method than by the float-out method (5965754 million sperm per vas deferens 3410876 million sperm per vas deferens, P< 0.05), indeed, the number was similar to that recorded for the ejaculated sperm (6303782 million sperm). The float-out was among the easiest techniques to perform, and although a low amount of sperm was obtained, there was a much lower incidence of extraneous cells in the sample, making it easier to find sperm. This technique was also the least likely to damage sperm and has the greatest likelihood for obtaining mature sperm, as only those sperm that was free in the lumen was included in the solution (Gunn et al., 2008). According to Salting et al. (2016), the concentrations of samples retrieved from the excurrent ducts, regardless of the retrieval method used, were found at the normal range in ejaculated samples.

Table 2. Comparison of two post-mortem sperm retrieval techniques of local cocks' in Eastern Algeria

Items	Float-out method	Flushing method	P value
N	6	6	
Volume (ml)	0.17±0.08	0.72±0.12	***
Concentration (10^6)	1.75±0.76	3.33±1.63	ns
Initial motility (%)	75,83±8.61	77.50±6.89	ns
Viability (%)	74.67±10.15	75.00±10.39	ns
Abnormality (%)	30.33±4.68	30.33±4.68	ns

Number (n),*** p<0.001 and no significant difference (ns)

Results impliedthat concentrations of spermatozoa obtained from the excurrent ducts were comparable with the concentrations of spermatozoa in samples obtained via dorso-abdominal massage method. The normal concentration of spermatozoa varies between individuals of the same species (Glover, 2012), it has been estimated between 1.7 and 3.5 billion spermatozoa per millilitre of chicken (Hicks, 1992), these numbers are consistent with the results found in this study.

As shown on the table 2, the motility of samples retrieved using the float-out was 75.83±8.61% and 77.50±6.89% for flushing method. T-test revealedno significant difference (P>0.05) between the motility of the float-out and the flushing retrieved spermatozoa indicating that the retrieval method did not significantly affect or improved the resulting percentage of motile and progressive spermatozoa in the sample collected. Which was higher (65.5±4.97%) than findings obtained by Salting et al. (2016) (swim-up method) and 65±4.71% (mincing method). Higher sperm motility has been observed in freshly ejaculated semen of cocks (66.67±6.67% to 86.5±0.78%) as reported in earlier studies (Almahdi and Ondho, 2014; Churchil et al., 2014). The flushing method affected more negatively than float out method on a percentage of sperm showing progressive motility (Villaverde-Morcillo et al., 2016). The float-out method, which has been used with the pukeko (Gunn et al., 2008) and mice (Mohammadzadeh et al., 2011), that was easier to perform and faster than the flushing method, but the sperm samples were usually contaminated with blood cells and urates, adversely affecting the long-term storage of the sperm, because the presence of white blood cells may increase the production of reactive oxygen species, according to Aitken and Bennetts (2006). Unlike the flushing method reduced contamination by blood cells and urates, according to Villaverde-Morcillo et al., (2016), and have shown that the flushing method was more effective. However, recovering roughly the same number of sperm is in a single vas deferens as in ejaculates.

Percentage of live spermatozoa in the sample retrieved by the float-out was found at 74.67±10.15% whereas, for flushing methods retrieved spermatozoa, viability was 75.00±10.39%. Statistical analysis shows no significant (P>0.05) difference between the viability of the float-out and flushing methods retrieved spermatozoa (Figure 3). Results indicated that the retrieval method had no effect on the resulting percentage of viable sperm in the sample. The viability of samples obtained in this study was lower than that obtained of the excurrent ducts by Salting et al. (2016), 85.65 ±7.88% (float-out method) and 84.20±8.79 % (mincing method), and to the results of these earlier studies (Tabatabaei et al., 2009; Tarif et al., 2013; Churchil et al., 2014). The percentage of live spermatozoa in the excretory ducts would be similar to that of ejaculated samples in domestic poultry because the spermatozoa mature and become fully fertile in the vas

deferens. It was also in the vas deferens where sperm produced by the testicles were stored until the rooster's companion (Salting et al., 2016). These differences may be explained by breed, location, nutrition, age and climate differences because the sperm parameters can vary depending on these factors (Das et al., 2016; Kuzlu and Taskin, 2017; Mavi et al., 2018).

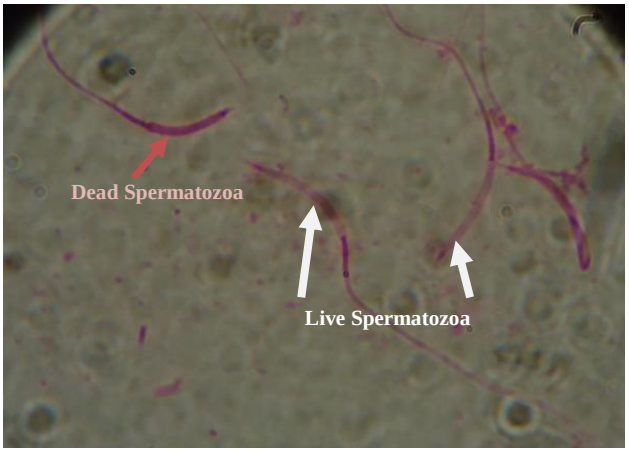


Figure 3. Assessment of sperm viability at local cocks' in Eastern Algeriawith eosin nigrosin (white: live spermatozoa, pink: dead spermatozoa)

As presented in table 2, the percentage of morphologically abnormal spermatozoa in samples retrieved by float-out was 30.33±4.68 % while 30.33±4.68 % abnormality was observed in flushing retrieved samples too. No difference was found between the percentages of abnormality of spermatozoa retrieved using float-out and flushing method. The percentages of abnormal spermatozoa observed in this study from vas deferens collected by both techniques (float-out and flushing method) were higher than those observed from ejaculated spermatozoa of various strains in studies conducted by Tarif et al. (2013) and Churchil et al. (2014), which ranged from 4.52±10% to 23.33±6.67% and lower than reported by Tabatabaei et al. (2009) in exotic Ross-308 and indigenous chicken in Iran ranged from 41.04±10.19% to 44.1±0.26%, respectively. Although this may be attributed to the genetic or intrinsic factors of Algerian local cocks, unfortunately, pieces of literature regarding the characterization of semen ejaculates of local breeds or strains were lacking. In this study, the isolation of spermatozoa from vas deferens of Algerian indigenous cocks was made possible and the difference between the quality of spermatozoa retrieved by float-out and flushing method was also studied. The flushing method allows for obtaining higher numbers of sperm. This technique can be easily performed on wild and domestic birds found dead or critically injured, which can be interesting in breeding and conservation programs. Many studies have shown that different factors include species, age, weight and others play significant roles in the differences in semen parameters (Salting et al., 2016)

A similar study of the harvest and storage specific to the specific strain for Algerian local chickens was recommended. In addition, other in vitro tests and subsequent correlation with cryo survivability have been suggested.

The effects of conservation the vas deferens at different temperatures

The effect of temperature on sperm quality during storage in situ, 2h after slaughter (20°C), 24h at 20°C and 24h at 4°C is shown in table 3. In this study, the quality of sperm samples obtained from vas deferens of local cocks, stored 24 h at 4ºC and at 20°C, after slaughtering was also determined. Holding spermatozoa at temperatures below body temperature prior cryopreservation was commonly done in many species with acceptable results. Since refrigeration devices were widely available, testes of animals could be kept at 4°C during its storage.

Table 3. Effects of conservation the vas deferens at different temperatures of localcocks' in Eastern Algeria

	2h	24h 4°C	24h 20°C	P
N	**6**	**3**	**3**	**value**
Initial motility (%)	81,00±2,19	55,00±5,00	83,00±7,21	***
Viability (%)	75,50±11,11	48,33±3,51	81,00±7,21	**
Abnormality (%)	32,00±4,65	32,00±4,36	29,67±2,52	ns
Acrosome integrity (%)	75,67±10,61	75,33±2,52	81,67±7,51	ns

Number (n), hours (h)** p<0.01 and *** p<0.001, no significant difference (ns)

In the present study, the motility of spermatozoa collected from the vas deferens, and conserved at 24 h in 4°C (55±5%), while, at 20°C (83±7.21%), were significantly higher (P<0.01),in comparison to that of control spermatozoa from non-refrigerated vas deferens (immediatelyafter slaughtering) (81±2.19%) and after conservation at 4°C. Unlike in present study, the motility decreases significantly (P<0.01) during *in-vitro* storage and after 24 h at 20°C, was seen 30.6 %. Semen samples stored at 5°C revealed an overall motility more than 50 % after 24 h (Mphaphathi et al., 2016), semen samples of the Venda breed record the motility of 55 % after 24h *in vitro*storage at 5°C while, semen samples stored at 25°C, caused a drastic reduction to 30 % after 24 h (Mphaphathi

et al., 2016). According to Clarke et al. (1982), cock semen stored at 41°C drastically decreased the sperm motility compared to that stored at 5, 15 and 25 °C. There were also significant differences concerning, the viability of the spermatozoa recovered from vas deferens stored at 4°C and at 20°C (48.33±3.51% and 81±7.21%). In contrast, abnormal spermatozoa (32±4.36%, 29.67±2.52%) and acrosome integrity (75.33±2.52%, 81.67±7.51%) did not obtain significant differences.

CONCLUSION

It was concluded that good quality of spermatozoa could be collected from vas deferens, and it is possible to preserve semen of Algerian local cocks at 4°C and 20°C for 24 hours. It is obtained that high quality semen from vas deferens of local cocks can be purposed for a further study on cryopreservation in order to create a gene bank of the local Algerian chicken cocks

DECLARATIONS

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Author's contribution

Bouzebda Afri Farida and Bouzebda Zoubir were mainly responsible for the tabulation of experimental data and article writing under the supervision of Majdoub Sara, Djaout Amel and Smadi Moustapha Adnane.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article.

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