RESEARCH ARTICLE

Role of Alanine, Arginine and Glutamine on Storage Capacity of Abattoir Derived Epididymal Buck Semen at Refrigerated Temperature

Hari O. Singh[1], Bhoopendra Singh[2], Rajesh Kumar[2]*, Sushant Srivastava[2], Jitendra P. Singh[3]

Abstract

The study was conducted on 48 pairs of abattoir-derived testicles from mature bucks, irrespective of breed. The testicles were collected and packed in plastic bags in an icebox at 4°C and immediately transferred to the laboratory. The testicles were randomly divided into six storage groups, *i.e.*, 6, 12, 24, 48, 72, and 96 hours; 8 pairs of testicles in each group and were preserved in the refrigerator at 4°C. After completion of storage time, testes of respective storage groups were cleaned with physiological saline solution, and the fascia, blood vessels, and sheath were removed using BP blade and thumb forceps. Epididymal semen was harvested, split into four aliquots and diluted with Tris Fructose-yolk-glycerol (TFYG) dilutor without and with additives, *viz.* alanine @ 25 mM, arginine @ 25 mM, or glutamine @ 50 mM. Ten straws from each diluted aliquot were filled, sealed, and placed in cold handling cabinet at 4°C for further 24 hours of storage. The sperm motility, viability and HOS reactivity were evaluated on dilution and again after 24 hours of cold storage of straws using standard procedures. There was a gradual and significant deterioration in sperm quality with increasing storage time of testes from 6 to 96 hours at 4°C. Inclusion of all three amino acid additives in TFYG dilutor, and glutamine in particular, significantly improved sperm motility, viability, and HOS reactivity on dilution and at 24 hours of cold storage following each interval of testicles' preservation. Therefore, epididymal semen of elite buck testes preserved at 4°C can be utilized for artificial breeding up to 12-24 hours following dilution with TFYG dilutor containing amino acid additives, particularly glutamine @ 50 mM.

Keywords: Abattoir testes, Additives, Buck, Cold storage, Epididymal semen.

Ind J Vet Sci and Biotech (2021): 10.21887/ijvsbt.17.3.11

Introduction

Antioxidant properties of amino acids may help to protect sperm cells against cold shock (Sangeeta *et al*., 2015). Amino acids with glycerol improves post-thaw sperm motility by virtue of its combined cryoprotective action in sperms of several species, including goat (Kundu, *et al.,* 2001); however, exact cryoprotective mechanism is yet not clear (Santiago-Moreno *et al*., 2019). Use of alanine as an additive gave higher post-thaw sperm recovery from goat epididymal semen (Kundu *et al.,* 2001) and in ejaculated buffalo spermatozoa (Sheshtaway *et al.,* 2008). Arginine promotes the motility of epididymal sperm by improving glycolysis rate, which elevates the rate of Adenosine-5′-triphosphate (ATP) and lactate generation in spermatozoa (Patel *et al*., 1998). Arginine plays an important role in the physiology of goat spermatozoa, enhances cell metabolism, and has a protective effect against lipid peroxidation (Srivastava *et al.,* 2000). Hassanpour *et al*. (2010) reported that low concentrations of L-arginine (0.001, 0.01 and 0.1 mM) after 45 or 90 min of incubation had little effect on ram epididymal sperm motion parameters. Glutamine probably shows cryoprotective action on the sperm membrane by preventing lipid peroxidation (Khlifaouia *et al.,* 2005). The literature on effect of cold storage of testes on epididymal sperm quality and inclusion of different additives in dilutor on sperm preservability of semen harvested at different storage intervals of testes at 4°C is meager. Therefore, the present study was aimed to evaluate the effect of different cold storage intervals of slaughtered

[1]Department of Animal Husbandry, Government of Uttar Pradesh, India.

[2]Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya, Uttar Pradesh, India.

[3]Department of Veterinary Medicine, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya, Uttar Pradesh, India.

Corresponding Author: Rajesh Kumar, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science & Animal Husbandry, Acharya Narendra Deva University of Agriculture and Technology, Kumarganj, Ayodhya, Uttar Pradesh, India, e-mail: dr-rajesh25@gmail.com

How to cite this article: Singh, H.O., Singh, B., Kumar, R., Srivastava, S., & Singh, J.P. (2021). Role of Alanine, Arginine and Glutamine on Storage Capacity of Abattoir Derived Epididymal Buck Semen at Refrigerated Temperature. Ind J Vet Sci and Biotech, 17(3): 51-55.

Source of support: Nil

Conflict of interest: None.

Submitted: 02/03/2021 **Accepted:** 29/06/2021 **Published:** 16/08/2021

© The Author(s). 2021 Open Access This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

buck testes and then adding amino acids in Tris extender on harvested epididymal sperm quality on dilution and after 24 hours of further storage at 4°C.

Materials and Methods

The experiment was carried out at deep-frozen semen (DFS) laboratory of the Department of Veterinary Gynaecology and Obstetrics of the College at ANDUAT, Ayodhya, UP, India. Forty eight pairs of testes (total 96) were collected immediately after slaughter of bucks, irrespective of breed, from local abattoirs of Faizabad and Sultanpur districts of UP and AL-Nafees Proteins Pvt Ltd, Mewat, Haryana. The testes were soon packed in plastic bags in ice chest at 4°C and immediately transferred to DFS lab. Testicles were randomly and equally divided into six storage groups, *i.e.*, 6, 12, 24, 48, 72 and 96 hours; 8 pairs of testicles in each group and stored at 4°C in the refrigerator. After completion of storage time, epididymal semen of respective groups were harvested in small graduated test tubes, pooled for each pair, re-divided into four equal aliquots, and then diluted at 35°C with Tris fructose-yolk-glycerol (TFYG) extender without and with three amino acid additives.

Four combinations of TFYG dilutor used were Control or C (TFYG alone), T1 (TFYG + alanine @ 25 mM), T2 (TFYG + arginine @ 25 mM), and T3 (TFYG + Glutamine @ 50 mM). Semen samples were extended at 35°C to a final concentration of 10 million sperm/ml with each extender and soon evaluated for sperm motility, viability and HOS reactivity. Moreover, 10 straws were filled, sealed, racked, and stored in a cold handling cabinet at 4°C for 24 hours and re-evaluated from each diluted aliquot.

The progressive sperm motility was assessed subjectively using a high power objective (40X) of a phase-contrast microscope (Olympus, Japan) with a warm stage at 37°C. The rectilinear forward movement of spermatozoa was regarded as progressive motility. The sperm viability was determined by eosin-nigrosin staining under a phase-contrast microscope and completely unstained sperm cells were considered as viable. The functional integrity of the plasma membrane of spermatozoa was assessed by a hypo-osmotic swelling test (HOST) (Ahmad *et al.,* 2014), following 1 hours of incubation of treated semen at 37°C using a phase-contrast microscope at 400X magnification. Spermatozoa with visible coiling of tail were considered to be HOS reactive (Khatun *et al.,* 2021) and were assumed to have intact plasma membrane.

The data were processed for analysis of variance (one-way ANOVA) followed by Tukey's Column statistics for significance by the Graph pad prism using Version 5.00 software. The differences with values of $p < 0.05$ were considered to be statistically significant. Pearson's correlations were worked out between sperm quality parameters of diluted and post-preserved semen.

Results and Discussion

The sperm quality parameters observed on dilution with TFYG extender without and with three amino acids and following 24 hours of further storage at 4°C of buck epididymal semen harvested at different cold storage intervals are depicted in Table 1. There was a progressive and significant drop in sperm quality parameters with respect to increased storage time of buck testes at 4°C from 6 hours to 96 hours. However, the differences in observations between 6 hours and 12 hours of storage were statistically non-significant for all three parameters. These observations concurred well with several of previous studies on epididymal semen of ram and buck (Mir *et al*., 2012; Sasaf *et al*., 2015; Ouennes *et al*., 2019; Patel *et al.,* 2021). Although cold storage can protect epididymal spermatozoa to some extent by delaying post-mortem changes, elongation of post-mortem time to epididymal sperm retrieval gradually alters the chemical composition and reduces pH of the epididymal lumen, which in turn deteriorate sperm quality. Wachida *et al.* (2019) also recorded a gradual drop in sperm quality of epididymal semen following storage of slaughtered ram testes at 5°C for different intervals as we used in the present study.

Further, a significant ($p < 0.05$) improvement in sperm motility, viability and HOS reactivity was recorded in T1, T_2 and T_3 extenders as compared to control, and T_3 in particular on dilution and even after 24 hours of refrigeration preservation of epididymal semen for each storage interval of testes. Moreover, the values of sperm motility and viability were reduced highly significantly ($p < 0.01$) after 24 hours of refrigeration of diluted semen as compared to values on dilution at each interval of testicular storage, but no appreciable changes were noted on HOS reactivity between dilution and 24 hours of storage at 4°C. These findings concurred well with Kulaksij *et al.* (2012). In the present study, the addition of selected amino acids (alanine @ 25 mM, arginine @ 25 mM and glutamine @ 50 mM) in TFYG medium significantly improved the storage ability of buck epididymal sperm, which might be due to charged molecule nature of amino acids. Higher sperm motility observed in the presence of alanine concurred with the findings of Kundu *et al.* (2001), who also recorded higher post-thaw recovery of epididymal buck sperm. Sheshtaway *et al.* (2008) reported dose-dependent effect of alanine on post-thaw recovery of buffalo spermatozoa. Patel *et al.* (1998) reported increased metabolic activity of bucks epididymal sperm cells, lactic acid accumulation and increase in pH of cell suspension at low concentration of arginine addition. Sadeghi *et al.* (2020) reported higher total motility in liquid semen preserved at 5°C (0, 24, 48 hours) than at 17°C. They mentioned refrigerated storage of goat sperm impaired sperm motility, mitochondrial membrane potential and response to oxidation as storage time increased.

Table 1: Effect of amino acid additives in Tris fructose-yolk–glycerol extender on sperm motility, viability and HOS reactivity of epididymal buck semen at different intervals of refrigeration preservation

Sperm parameter	Storage time of testes (4°C)	*Epididymal semen on dilution at 35°C in*				*Epididymal semen after 24 hr of storage at 4°C*			
		TFYG Control	*TFYG + Alanine*	*TFYG + Arginine*	*TFYG + Glutamine*	*TFYG Control*	*TFYG + Alanine*	*TFYG + Arginine*	*TFYG + Glutamine*
Sperm motility (%)	6h	75.12 ± 0.58^{aA}	79.00 ± 1.15^{aB}	80.13 ± 0.52^{aBC}	81.75 ± 0.45^{aC}	39.38 ± 1.46^{aA}	44.75 ± 1.84^{aB}	44.88 ± 1.67^{aB}	49.00 ± 0.63^{aC}
	12h	73.75 ± 0.45^{aA}	76.00 ± 0.46^{aB}	77.50 ± 0.60^{aBC}	79.25 ± 0.56^{aC}	33.25 ± 0.82^{bA}	35.88 ± 0.91^{bB}	38.63 ± 0.60^{bC}	42.38 ± 0.50^{bD}
	24h	65.50 ± 0.36^{bA}	68.25 ± 1.19^{bB}	69.13 ± 0.83^{bC}	70.88 ± 0.85^{bC}	32.75 ± 0.80^{bA}	35.63 ± 0.80^{bB}	36.50 ± 0.78^{bBC}	37.50 ± 0.78^{cC}
	48h	59.00 ± 0.50^{cA}	62.63 ± 0.26^{cB}	63.75 ± 0.37^{cB}	66.50 ± 0.68^{cC}	23.63 ± 1.07^{cA}	26.38 ± 1.05^{cB}	27.75 ± 1.16^{cB}	32.13 ± 0.81^{dC}
	72h	48.25 ± 1.03^{dA}	52.13 ± 0.91^{edB}	53.50 ± 0.87^{dBC}	55.38 ± 0.75^{dC}	18.38 ± 1.23^{dA}	20.63 ± 1.48^{dAB}	22.50 ± 1.21^{dBC}	24.63 ± 0.94^{eC}
	96h	35.63 ± 0.63^{eA}	38.13 ± 2.16^{eA}	42.38 ± 1.47^{eB}	44.88 ± 1.42^{eB}	12.50 ± 0.50^{eA}	15.38 ± 0.57^{eB}	16.38 ± 0.57^{eBC}	17.38 ± 0.57^{fC}
Sperm viability (%)	6h	81.50 ± 0.50^{aA}	85.63 ± 0.60^{aB}	86.63 ± 0.38^{aB}	88.25 ± 0.25^{aC}	66.63 ± 1.11^{aA}	69.00 ± 1.16^{aAB}	70.38 ± 1.05^{aB}	72.25 ± 0.96^{aC}
	12h	80.88 ± 0.40^{aA}	82.88 ± 0.55^{aB}	84.75 ± 0.67^{aC}	87.00 ± 0.53^{aD}	64.13 ± 0.40^{aA}	66.63 ± 0.65^{aAB}	68.25 ± 0.88^{aB}	72.13 ± 0.69^{aC}
	24h	72.25 ± 0.80^{bA}	75.25 ± 0.96^{bB}	78.38 ± 1.10^{bC}	81.88 ± 0.95^{bD}	59.50 ± 0.93^{bA}	61.38 ± 0.89^{bAB}	62.38 ± 0.89^{bB}	63.38 ± 0.89^{bB}
	48h	68.00 ± 0.76^{cA}	73.88 ± 0.72^{bB}	75.25 ± 0.65^{bBC}	79.00 ± 0.87^{bC}	39.13 ± 1.51^{cA}	42.00 ± 1.70^{cA}	46.38 ± 1.73^{cB}	51.25 ± 1.75^{bC}
	72h	56.75 ± 0.86^{dA}	64.38 ± 1.25^{cB}	66.50 ± 1.23^{cBC}	68.88 ± 1.13^{cC}	29.00 ± 1.23^{dA}	31.75 ± 1.39^{dAB}	32.38 ± 1.40^{dBC}	34.38 ± 1.19^{cC}
	96h	44.00 ± 0.60^{eA}	54.13 ± 0.91^{dB}	55.63 ± 0.78^{dC}	57.63 ± 0.78^{cC}	20.50 ± 0.63^{eA}	22.50 ± 0.63^{eAB}	23.50 ± 0.63^{eB}	24.50 ± 0.63^{dB}
HOS reactivity (%)	6h	36.63 ± 0.57^{aA}	37.88 ± 0.52^{aAB}	39.88 ± 0.68^{aBC}	41.63 ± 0.78^{aC}	34.75 ± 0.80^{aA}	39.25 ± 1.53^{aB}	42.00 ± 1.28^{aBC}	44.25 ± 1.25^{aC}
	12h	34.38 ± 0.50^{aA}	36.00 ± 0.73^{aAB}	38.00 ± 0.65^{aC}	39.50 ± 0.65^{aC}	32.75 ± 0.92^{aA}	36.63 ± 0.63^{aB}	38.63 ± 0.63^{bBC}	40.63 ± 0.63^{bC}
	24h	25.88 ± 0.81^{bA}	29.00 ± 1.03^{bB}	30.25 ± 0.84^{bBC}	32.25 ± 0.82^{bC}	23.63 ± 0.71^{bA}	27.13 ± 0.99^{bB}	29.50 ± 0.94^{cBC}	32.13 ± 1.21^{cC}
	48h	18.75 ± 0.67^{cA}	20.13 ± 0.77^{cB}	22.00 ± 0.70^{cB}	24.00 ± 0.65^{cC}	20.00 ± 0.53^{cA}	20.63 ± 0.94^{cAB}	22.63 ± 0.94^{dAB}	24.63 ± 0.94^{dB}
	72h	11.75 ± 0.37^{dA}	15.00 ± 0.42^{dB}	13.75 ± 0.37^{dBC}	15.25 ± 0.53^{dC}	11.13 ± 0.48^{dA}	14.38 ± 0.46^{dB}	16.38 ± 0.46^{eC}	18.38 ± 0.46^{eD}
	96h	9.50 ± 0.63^{dA}	11.25 ± 0.45^{eAB}	12.88 ± 0.40^{dBC}	14.38 ± 0.60^{dC}	8.75 ± 0.65^{eA}	10.38 ± 0.50^{eAB}	12.38 ± 0.50^{fBC}	14.38 ± 0.50^{fC}

Means bearing uncommon superscripts in a column (lower case) and row (upper case) differ significantly ($p < 0.05$) for each attributes.

Table 2: Correlation coefficients (r=value) of sperm quality parameters in post-dilution and post-refrigerated epididymal buck semen in Tris extender (6 hours)

Parameters	*PD Motility %*	*PD Live sp. %*	*PD HOST %*	*PP Motility %*	*PP Live sp. %*
PD Live sperm %	0.96**				
PD HOST %	0.93**	0.94**			
PP Motility %	0.85**	0.84**	0.90**		
PP Live sperm %	0.91**	0.96**	0.83**	0.72**	
PP HOST %	0.97**	0.89**	0.92**	0.87**	0.80**

PD = post-dilution, PP = Post-preservation in Tris with amino acids, **Significant at $p < 0.01$.

The percentage of viable cells normally exceeds that of motile cells; therefore it is clinically important to know whether immotile sperms are alive or dead. Viability results should be assessed in conjunction with motility results from same semen sample. The presence of a large proportion of vital but immotile cells may indicate structural defects in the flagellum (Chems and Rawe, 2003). High HOS reactive spermatozoa in amino acids treated group may be due to membrane stabilizer action of these amino acids on spermatozoa. Similar finding was also observed by de Mercado *et al. (*2009) in boar semen.

In present study, significantly ($p < 0.05$) higher sperm motility and viability observed after 24 hours of storage of semen in glutamine treated group compared to alanine, arginine and control might be due to variable degree of membrane stabilizing property of these amino acids (Kundu *et al.,* 2001; Khlifaouia *et al.,* 2005), however, the exact mode of action yet is not clear. Furthermore, Kruuv *et al.* (1998) and Trimeche *et al.* (1998) reported toxic effect of higher glutamine concentration (152.7 mM and 240 mM, resp.) on mammalian sperm cells by virtue of its osmotic effect and biochemical toxicity and concluded that the sensitizing effect of the hyper-tonicity neutralizes the protective effect of glutamine. The membrane integrity (HOS reactivity) was recorded higher in all amino acids treated groups in our study. It may be due to protective role of amino acids on cauda epididymal spermatozoa. The variation observed in semen quality in different studies might be due to the difference in concentration of amino acids used and thereby altered osmolarity and toxicity of the medium.

Pearson's correlations studied among three vital sperm quality parameters at 6 hours of cold storage stage of testes between post-dilution and post-preservation (for 24 hours at 4°C) in tris extender with amino acids additives (Table 2), as expected physiologically, revealed highly significant ($p < 0.01$) positive interrelationships among sperm motility, viability and HOS reactivity on dilution ($r = 0.93$-0.96) and at post-refrigeration preservation ($r = 0.72$-0.87) stage. Moreover, the values of all three parameters on dilution of epididymal semen had significant ($p < 0.01$) positive correlations with values on post-refrigeration of diluted semen for 24 hours ($r = 0.83$-0.97). The magnitudes of correlations were somewhat higher in samples extended in TFYG dilutor with amino acids as compared to control dilutor on dilution, post-refrigeration as well as between post-dilution and post-refrigeration periods. These correlations suggest that the initial sperm quality determines the storage capability of semen at 4°C temperature.

Conclusion

From the study, it can be concluded that the testes as whole or epididymal semen can be preserved satisfactorily at 4°C up to 12-24 hours of the slaughter of elite buck and further utilized for breed improvement in goat. The addition of glutamine @ 50 mM in TFYG dilutor significantly improves motility, viability and HOS reactivity of epididymal buck semen up to 24 hours of storage at 4°C; however, its effect on *in vitro* fertilization and embryo production is still to be explored.

Acknowledgement

We thank the Dean, CVSc & AH, and authorities of ANDUAT, Ayodhya for fund and facilities provided to conduct this work.

References

Ahmad, M., Nasrullah, R., Riaz, H., Sattar, A., & Ahmad, N. (2014). Changes in motility, morphology, plasma membrane and acrosome integrity during stages of cryopreservation of buck sperm. *Journal of the South African Veterinary Association*, *85*(1), 972.

Chemes, H.E., & Rawe, Y.V. (2003). Sperm pathology: a step beyond descriptive morphology. Origin, characterization and fertility potential of abnormal sperms phenotypes in infertile men. *Human Reproduction Update, 9*, 405-428.

de Mercado, E., Hernandez, M., Sanz, E., Rodriguez, A., Gomez, E., Vazquez, J. M., Martinez, E. A., & Roca, J. (2009). Evaluation of l-glutamine for cryopreservation of boar spermatozoa. *Animal Reproduction Science*, *115*(1-4), 149-157.

Hassanpour, H., Teshfam, M., Goodarzi, A.K., Tajik, P., & Mirshokrai, P. (2010). In vitro effects of l-arginine on motion parameters in ram epididymal sperm. *Comparative Clinical Pathology, 19,* 351-355.

Khatun, A., Fazili, M.R., Malik, A.A., Shah, R.A., Khan, H.M. , Choudhury, A.R., Naikoo, M., Lone, F.A., & Malik, A. (2021). *In vitro* assessment of tris egg yolk and soybean lecithin based extenders for cryopreservation of crossbred ram semen. *CryoLetters*, *42*(2), 73-80.

Khlifaoui, M., Battut, I., Bruya, J.F, Chatagnon, G., Trimeche, A., & Tainturier, D. (2005). Effects of glutamine on post-thaw motility of stallion spermatozoa: an approach of the mechanism

of action at spermatozoa level. *Theriogenology*, *63*, 138-149.

Kruuv, J., Glofcheski, D.J., & Lepock, J.R. (1998). Protective effect of L-glutamine against freeze-thaw damage in mammalian cells. *Cryobiology, 25*, 121-130.

Kulaksiz, R., Cebi, C., & Akcay, E. (2012). The effect of different extenders on the motility and morphology of ram sperm frozen or stored at 4°C. *Turkish Journal of Veterinary and Animal Sciences, 36*(2), 177-182.

Kundu, C.N., Das, K., & Majumder, G.C. (2001). Effect of amino acids on goat cauda epididymal sperm cryopreservation using a chemically defined model system. *Cryobiology, 41*, 21-27.

Mir, S.S., Lone, F.A., Khan, M.Z., Malik, A.A., Islam, R., & Sofi, K.A. (2012). Effect of cold storage period on the quality of ram cauda epididymal spermatozoa recovered post-mortem. *Turkish Journal of Veterinary and Animal Science, 36*(6), 683-687.

Ouennes, H., Bouzebda, F.A., Bouzebda, Z., Medjedoub, S., Djout, A., & Smadi, M.A. (2019). Effect of testicle post-mortem storage on goat epididymal sperm quality: the first step towards cryobank for local Algeria breeds. *Revue de Médecine Vétérinaire*, *170*(7-9), 184-192.

Patel, A.B., Srivastava, S., Phadke, R.S., & Govil, G. (1998). Arginine activates glycolysis of goat epididymal spermatozoa: An NMR study. *Biophysical Journal,* 75, 1522-1528.

Patel, R.P., Singh, B., Kumar R., & Srivastava, S. (2021). Biometry and storage ability at 4°C of slaughtered buck testis and correlations of various epididymal seminal attributes. *The Indian Journal of Veterinary Sciences and Biotechnology, 17*(2), 72-77

Sadeghi, S., Del Gallego, R., García-Colomer, B., Gómez, E.A., Yániz, J.L., Gosálvez, J., López-Fernández, C., & Silvestre, M.A. (2020). Effect of sperm concentration and storage temperature on goat spermatozoa during liquid storage. *Biology*, *19*(9), 1-13.

Sangeeta, S., Arangasamy, A., Kulkarni, S., & Selvaraju, S. (2015). Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. *Animal Reproduction Science*, *161,* 82-88.

Santiago-Moreno, J., Bernal, B., Perez-Cerezales, S., Castano, C., Toledano-Díaz, A., Esteso, M.C., Gutierrez-Adan, A., Lopez-Sebastian, A., Gil, M.G., Woelders, H., & Blesbois, E. (2019). Seminal plasma amino acid profile in different breeds of chicken: Role of seminal plasma on sperm cryoresistance. *PloS one*, *14*(1), e0209910.

Sasaf, B., BelKadi, S., Belkacem, L., Mamache, B., & Tlidjane, M. (2015). Variations of motility and survival with storage time at 4° of epididymal spermatozoa of Ouled-Djellal breed rams in Eastern Algeria. *Veterinary World*, *8*(2), 326-329.

Sheshtawy, R.I., El-Sisy, G.A., & El-Nattat, W.S. (2008). Use of selected amino acids to improve buffalo bull semen cryopreservation. *Global Veterinarian, 2*, 146-150.

Srivastava, S., Desai, P., Coutinho, E., & Govil, G. (2000). Protective effect of L-arginine against lipid peroxidation in goat epididymal spermatozoa. *Physiological Chemistry and Physics and Medical NMR*, *32*(2), 127-135.

Trimeche, A., Renard, P., & Tainturier, D. (1998). A procedure for Poitou jackass sperm cryopreservation. *Theriogenology*, *50*(5), 793-806.

Wachida, N., Bassey, U.E., & Dawuda, P.M. (2019). Effect of storage time on the quality of cauda epididymal spermatozoa of West African dwarf (WAD) rams. *Animal Reproduction Science, 205,* 144-149.