Egyptian Journal of Veterinary Sciences
https://ejvs.journals.ekb.eg/

Effects of Thawing Methods (Techniques) on Freeze Thaw Buffalo Bull Sperm Quality

Rajbari Rajbari[1], Asma U Husna[2], Asima Azam[3], Rabea Ejaz[3], Rooma Adalat[1], Shamim Akhter[4] Muhammad U Farooq[5], Saima Qadeer[5]*

[1]Department of Zoology, University of Sialkot-51310, Punjab- Pakistan

[2]Department of Biology, University of Haripur, 22620, Khyber Pakhtunkhwa- Pakistan

[3]Department of Zoology, Shaheed Benazir Bhutto Women University Peshawar- 25000, Pakistan

[4]Department of Zoology, Wildlife and Fishries, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-46300, Pakistan

[5]Department of Zoology, Division of Science and Technology, University of Education, Lahore-54000 Pakistan

BACKGROUND: Semen cryopreservation involves preserving the sperms so they can be used later. This technique can prove to be a pathway for restoring the number of endangered species as their sperm can be preserved and used for fertilization later on.

Objective: The propose of study is to find out the optimum thawing time, temperature and post thaw incubation period of cryopreserved buffalo sperm.

Methods: Three buffalo bulls of the 1-2 year age were selected at Semen Production Unit, Qadirabad, Pakistan. Semen was collected by artificial vagina at 42 °C and 18 qualified ejaculates (two ejaculates/bull/week) for 3 weeks (replicate) were cryopreserved following a standard protocol. Frozen semen was divided in different groups, with each group being thawed at different temperature and time. Group I was subjected to slow thawing at 4-5°C, 2nd group to moderate thawing at 35ºC-37 ºC , 3rd group to rapid thawing while the 4th one was subjected to air thawing. Moreover, optimum incubation period of cryopreserved sperm was evaluated by thawing and incubating 42 straws in a water bath at 37ºC in with 2 straws being assessed every hour up till 10 hours.

Results: Thawed semen samples were assessed based on different semen quality parameters. Acrosome integrity, plasma membrane integrity and progressive motility of cryopreserved buffalo sperm were significantly higher ($P<0.05$) in Group II which was subjected to moderate thawing. However, sperm quality parameters remained same ($P > 0.05$) with different incubation time (from 1 hr to 11 hr at 37°C).

Conclusion: In conclusion, moderate thawing is effective in protecting the sperm cell from freeze thaw damages during cryopreservation. Sperm functional quality parameters were very acceptable after even 5 h of incubation indicated the usefulness of these incubated semen straws for field insemination in areas where availability of liquid nitrogen containers is problematic. Digital thermos maintained at 37°C could be alternative for short distance travelling for taking semen doses at insemination site. Further research efforts are needed to verify the fertilizing ability of this straw incubated (37°C) semen.

Keywords: Buffalo, Cryopreservation, Semen, Thawing Protocols,

Corresponding author: Saima Qadeer, E- mail.: saima.qadeer@ue.edu.pk., Tel. +923315013310

(*Received* 30/05/2022; *accepted* 21/08/2022)

DOI. 10.21608/EJVS.2022.130902.1333

©2022 National Information and Documentation Centre (NIDOC)

Introduction

Cryopreservation is a process that takes into account a high level adaptation of living cells to thermal and osmotic shock. The osmotic and thermal stress results in the loss of acrosome integrity, sperm plasma membrane integrity and damages the nucleus as well [1]. Sperm motility, along with the aforementioned parameters of spermatozoa are the essential factors that ensure successful artificial insemination (AI) [2]. The type of extender, cryoprotectants used, packaging system, freezing procedure and thawing time are some of the important factors that affect sperm quality after thawing [3-5].

Thawing technique has the same importance as the freezing one as far as its effect on sperm survival is concerned during cryopreservation [6]. Thawing temperature needs to be carefully monitored in order to avoid any damage to the sperm. During the cryopreservation process, a number of factors affect the thawing rate. These include type of extender, glycerol concentration and packaging material [7]. Experimental protocols, tools and chemicals may vary from country to country and even within different areas of the same country. Pakistan is among those under developed countries where the number of AI units is limited and an AI technician has to deal with a wide range of issues with scarce funds and resources. These AI technicians carry semen in liquid nitrogen tanks while performing their duties at farmer's doorstep. It is a tough job to do as handling of liquid nitrogen containers is not easy and requires expertise in the field. Moreover, liquid nitrogen gas can evaporate during travelling which affects the overall semen quality. So, it is the need of the hour to introduce a relatively inexpensive and easy method of carrying the semen without liquid nitrogen containers. Not much work is done and there are only a limited number of studies regarding the effects of field-handling practices on semen quality which in turn alters the fertility of frozen semen straws. Therefore, current study was planned to compare the effect of different thawing methods being practiced on the quality of buffalo sperm. It was also one of the objectives to determine a suitable thawing method that is economical as well as feasible for the AI technicians to take the semen straws in the field without carrying the load of bulky liquid nitrogen tank.

Material and Methods

Extender preparation

Extender was prepared from *Tris*-Citrate buffer (having a pH 7.0, osmotic pressure of 320 mOsmol kg^{-1}) The ingredients of this buffer were 1.56g citric acid and 3.0g tris-(hydroxymethyl-aminomethane), glycerol 7%, fructose 0.2% w/v, egg yolk 20% v/v, antibiotics (benzyl penicillin 1000 IU mL^{-1} and streptomycin sulphate 1000 µg mL^{-1}) which were dissolved in 74mL distilled water [4].

Semen Collection and Initial Evaluation

This study involved the collection of semen ejaculates (two ejaculates/bull/week) for 3 weeks (replicate) from 3 Nili-Ravi buffalo bulls (kept at Semen Production Unit Qadirabad) of the 1-2 year of age by using an artificial vagina at 42˚C. Collected semen was then instantly transferred to the laboratory where it was observed under 40X microscope for the assessment of sperm motility at 37˚C. Concentration of the sperm was analyzed using Bovine Photometer (IMV technologies Accucell). The semen samples meeting the criteria of (>0.5 ml volume, >0.5 × 10^9 sperm ⁄ ml concentration, >60% motility) were selected for further processing [5].

Semen Extension and Cryopreservation

Dilution of semen aliquots was done in a single step at 37˚C at the rate of 5.0×105 motile sperms mL-^{1}. This extended semen was then cooled down to 4˚C in 2 hours and equilibrated at 4˚C for 4 hours. This processed semen was then filled up in 0.5 mL French straws by using the suction pump at 4˚C in the cold cabinet and stored in liquid nitrogen vapors for 10 minutes. Straws were immersed and preserved into liquid nitrogen (-196˚C). After 24 hours of storage two semen straws were taken out and thawed through each method given below. Each of these straws was then assessed for post thaw semen quality [8].

Thawing procedures:

Following methods were employed for thawing purpose:

Group I. (Slow Thawing; ST):

1. 4ºC - 5°C for 35 min
2. Ice cubes thawing for 30 min and
3. Ice cubes thawing 60 min

Group II. (Moderate thawing; MT):

1. 35ºC for 12 sec
2. 37°C for 15 sec

3. 37°C for 20 sec
4. 37°C for 30 sec and
5. 37°C for 45 sec

Group III. (Rapid thawing; RT):
1.50ºC for 15 sec
2. 60°C for 8 sec
3. 70ºC for 7 sec

Group IV. (Air thawing; AT):

For air-thawing straws were taken out from the liquid nitrogen storage vessel and kept in open air for 30 seconds. Semen samples were then subjected to post thaw semen quality assessment after thawing. Environmental temperature during the study period was kept around 35-40°C.

Effect of Incubation Period

A total of 42 straws were thawed in a water bath at 37°C to assess the incubation period effect on cryopreserved semen. The assessment was done without cutting the straws in such a way that every hour, 2 straws were taken out for assessment of semen quality and it continued up till 10 hours.

Post Thaw Semen Evaluation

Progressive Linear Motility

Sperm motility was evaluated by placing 10µL of semen on a glass slide which was then observed under 40X phase contrast microscope.

Plasma Membrane Integrity

Plasma membrane integrity of buffalo bull sperm was assessed using Hypo osmotic swelling assay (HOS). HOS assay solution consisted of sodium citrate (0.73g) and fructose (1.35g) in 100 ml distilled water (osmotic pressure ~190 mOsmol Kg-1). 50µl of frozen thawed semen was then mixed up with 500µl of HOS solution and incubated for 30-40 min at 37°C for evaluation of sperm tail plasma membrane integrity. Again, 10 µl of semen sample was placed on glass slide, covered with coverslip and observed under 40X phase contrast microscope. Two hundred spermatozoa per replicate were counted for their swelling, considering coiled tail representing undamaged sperm plasma membrane [8].

Acrosome Integrity and normality

100µl semen was mixed up with 500 µl of 1% formal citrate in order to evaluate the acrosome intactness. A drop of the resulting of mixture was then placed in the middle of the slide covered with cover slip. Integrity was categorized by normal apical ridge and normality was observed under 100X by placing oil immersion value.

Statistical Analysis

ANOVA was used for the analysis of data on semen quality parameters. LSD test was used for the comparison of means once F-ratio was found significant.

Results

Table 1 is reflecting the data about the effect of different thawing temperatures on buffalo sperm motility. Figure 1 is depicting that group II is significantly different from the rest of the groups on the basis of quality of post thaw semen. Progressive sperm motility remained comparatively unaltered ($P > 0.05$) throughout the exposure time within Group II which was subjected to moderate thawing from 35°C-37°C as shown in Fig. 1 and Table 2. The slow, rapid and air thawing protocols didn't reflected any

TABLE 1. Effect of different thawing temperatures on post-thaw progressive motility of buffalo bull spermatozoa.

Thawing Temperature	Sperm Motility (%)
Slow Thawing	
4ºC - 5°C for 30- 40 min ice water	11.66±6.66
Ice thawing for 30 minutes and 60 minutes	10±2.88
Rapid Thawing	
50ºC for 15 seconds	21.66±6 (circular motility)
60°C for 8 seconds	21.66±1.66 (circular motility)
70ºC for 7 seconds	0.0±0
Air Thawing	13.33±1.66

Values are Mean± Mean± Standard Error

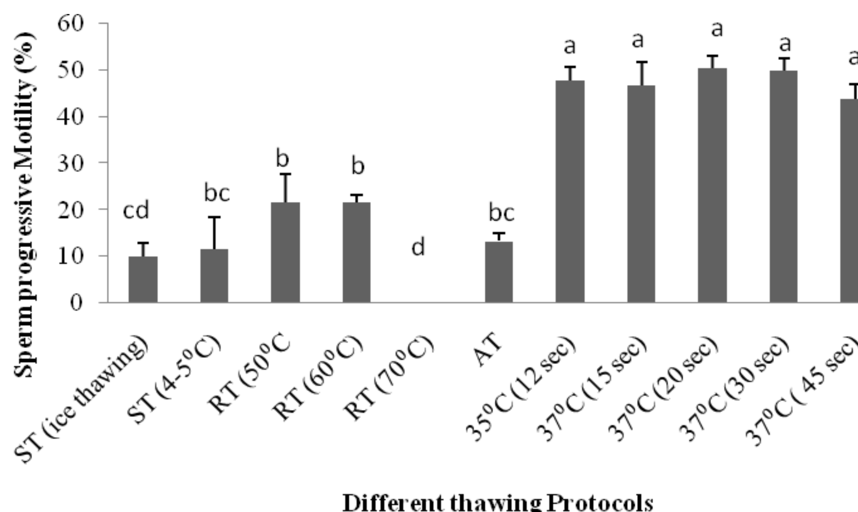

Fig. 1. Effect of different thawing techniques on post thaw sperm motility (Mean ± SE) in buffalo bull spermatozoa. Bars reflect significant difference from each other (P<0.05). (ST: Slow thawing, RT: Rapid thawing, AT: Air thawing).

significance that could qualify them for the further assessments of sperm functional parameters as the sperms did not survived these thawing procedures and progressive motility was not observed in these groups.

Table 2 is showing the data on the effect of a range of moderate thawing temperatures on post-thaw sperm plasma membrane integrity(%), acrosome integrity(%), progressive motility and normality. Plasma membrane integrity (expressed in percentage) of sperm was comparable (P > 0.05) from 35⁰C (12 sec) to 37⁰C (15, 20, 30, 45 sec) in Group II.

Furthermore, Table 2 is also showing impact of optimum thawing temperatures on post-thaw acrosome integrity and normality of sperm. As per the results obtained, no significance(P > 0.05) was observed between acrosome integrity and normality of sperm at moderate thawing temperature range of 35⁰C-37⁰C for variable time periods.

Table 3 is presenting the data on the effect of incubation period (hrs) at 37°C on different sperm quality parameters such as sperm plasma membrane integrity, post-thaw sperm progressive motility, acrosome integrity and normality of buffalo sperm. Very encouraging results were obtained from this experiment as the sperm quality parameters such as those of acrosome integrity, plasma membrane integrity, progressive motility, and normality remain unaltered and the sperms remained viable in the semen samples incubated for 1 hr to 11 hr at 37°C.

Discussion

The current study was conducted for the comparison of different thawing procedures as well as to find out the effect of incubation period at 37°C on sperm fertilizing ability. It involved recording multiple sperm parameters such as intactness of acrosome and plasma membrane, progressive sperm motility, and number sperm count in order to estimate the freeze thaw sperm quality of Nili-Ravi buffalo bull. The results reflected that sperm samples thawed at mild or average temperatures were quite efficient in maintaining the aforementioned sperm parameters in a healthy state unlike those that were subjected to slow, rapid and air thawing procedures.

It has also been reported that the long exposure of semen to the extreme temperatures results in pH alterations and halts the functioning of proteins that ultimately leads to sperm death [9]. As per the results, the spermatozoa lost progressive motility and all the groups exhibited low sperm motility except the group II. Instead spermatozoa in these groups exhibited circular motility. These findings suggest that extreme thawing temperatures have a negative effect on the sperm motility. Therefore, only Group II was considered eligible for further analysis of sperm parameters.

Our findings are in agreement with Holt (2000) who concluded that despite rapid thawing minimizes the chance of water recrystallization

TABLE 2. Effect of moderate thawing temperature on different sperm parameters.

(Different thawing temperatures)	Sperm Progressive Motility (%)	Plasma Membrane Integrity (%)	Acrosomal Integrity (%)	Normality (%)
35°C (12 sec)	47.8±2.935	61±3.605	71±4.163	91.33±1.232
37°C (15 sec)	46.7±5.024	66±3.385	61.66±1.714	88.44±1.634
37°C (20 sec)	50.6±2.412	62.76±3.428	60.9±2.664	93.07±0.867
37°C (30 sec)	50±2.54	63.66±1.356	71.2±6.575	90.89±1.522
37°C (45 sec)	43.9±3.089	61.366±1.202	69±7.002	90.48±0.962

Values are Mean± Standard Error All values are non-significant (P > 0.05).

TABLE 3. Data on the effect of incubation hours at 37°C on different sperm parameters.

Sperm Quality Assays	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	8 hr	9 hr	10 hr	11 hr
Sperm Progressive Motility (%)	55±0.0	53.3±1.7	51.7±1.7	48.3±1.7	51.7±1.7	51.7±1.7	50±2.9	48.3±1.7	48.3±1.7	46.7±4.4	45±5.8
Plasma Membrane Integrity (%)	67.7±0.3	65.0±0.6	67.3±2.6	60.7±2.6	58.3±2.3	63.7±2.7	60.0±6.5	57.0±6.7	54.7±6.0	58.0±7	57.3±6.2
Acrosome Integrity (%)	51.3±0.9	50.0±0.6	53.0±0.3	49.3±0.3	49.3±0.6	50.3±0.3	51.0±0.7	49.3±0.3	46.3±0.7	39.7±0.3	40.3±0.3
Normality (%)	84.3±0.7	80.0±0.6	78.7±0.7	79.7±0.9	77.0±1.2	74.7±0.9	73.7±0.9	71.7±0.9	70.3±0.3	74.3±0.3	73.3±0.9

Values are Mean±S.E. All values are non-significant (P > 0.05).

but at the same time it also damages the sperm cell membrane [10]. However some studies also gave contrary results as well as their results depict that rapid thawing is more productive in preventing the damage during rewarming[11,12]. This study also describes that slow thawing causes more damage to the sperm as compared to the rapid thawing process. Similar results were reported by some of the previous studies as well which were of the view that the main concern of the slow thawing technique is the appearance of more evident osmotic pressure changes[13,14]. Furthermore, thawing at higher temperate resulted in death of spermatozoa. Our results have also been supported by the studies done on boar and ram [15, 16].

In present study, thawing at 30-37°C for 30 seconds was found to be very effective for obtaining the high quality buffalo bull sperm. These results are supported by earlier studies as well which recommended thawing at 30°C -37°C for 30 sec as the optimum method for getting the buffalo bull sperm with high percentage of post thaw motility and integrity [17]. Similar results were reported by some of the previous studies as well which revealed that temperature range of 33°C-37°C with a thawing duration of 30-40 sec is most reliable protocol to be used for thawing[18,19].

The key outcome of second part of the study is that different parameters of sperm quality remained consistent even after 11 hours of incubation of straws at 37°C. This retained functionality could be attributed to the fact that the antioxidant activity of the sperm enzymes recovers faster at this optimum temperature. It is demonstrated earlier that there are two factors during the thawing process that regulate the extent to which the cell could be damaged, the degree of free oxygen radical production and the recovery rate of antioxidant activity. It is estimated that this recovery rate is faster than free oxygen radical production at 37°C. As a result of which, sperm attains the ability to nullify free

oxygen radical production efficiently but only when the temperature during incubation is highly controlled (37°C) prevent cell damage [20]. Maintaining suitable environmental conditions is very effective in the quality of fertility and preservation of sperm [21-26].

Conclusion

This study analyzed different thawing strategies to find out the level of their effectiveness in preserving the sperm. It is concluded that the spermatozoa thawed at moderate temperatures of 35ºC for 12 sec and 37°C for 15, 20, 30 and 45 sec (Group II) were the most effective to resist the damage of thawing process, opposite being true for Groups I, III and IV. It is highly recommended to educate the AI technicians and local farmers to strictly follow the thawing protocols during insemination of buffalo to ensure maximum conception rate. Secondly semen quality parameters of incubated straws can withstand even after 11 hours of incubation at 37°C. This could be a breakthrough point as it could help in taking the incubated straws in the field for insemination purpose by maintaining the temperature at 37°C. This temperature could be maintained by using digital thermos and is lot easier than carrying the liquid nitrogen containers to field.

Acknowledgments

The authors thank the Semen Production Unit Qadirabad for technical.

Conflict of Interest Statement

The authors declare no conflict of interest.

Data Availability Statement

All data is presented in this article. The data that support the findings of this study are available from the corresponding author upon request.

Authorship Statement

AUH, R and SQ were involved in planning the study. AA, RE and RA were involved in data analysis and drafting of the manuscript. R, AA and RA were involved in practical work and drafting of the manuscript. All authors read and approved the final manuscript.

References

1. Blesbois, E. Current status in avian semen cryopreservation. *World's Poultry Science Journal*, **63**(2), 213-222(2007).

2. Correa, J. R., Pace, M. M. and Zavos, P. M. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology,* **48**(5), 721-731(1997).

3. Ansari, M. S. Effect of L-Cysteine in Tris-citric egg yolk extender on post-thaw quality of Nili-Ravi Buffalo (Bubalus bubalis) bull spermatozoa. *Pakistan journal of zoology,* **43**(1), 41-47(2011).

4. Ansari, M. S., Rakha, B. A., Ullah, N., Andrabi, S. M. H., Iqbal, S., Khalid, M. and Akhter, S. Effect of exogenous glutathione in extender on the freezability of Nili-Ravi buffalo (Bubalus bubalis) bull spermatozoa. *Anim. Sci. Pap. Rep.,* **28**(3), 235-244(2010).

5. Andrabi, S. M. H. Factors affecting the quality of cryopreserved buffalo (Bubalus bubalis) bull spermatozoa. *Reproduction in Domestic Animals,* **44**(3), 552-569(2009).

6. Mazur, P. and Cole, K. W. Influence of cell concentration on the contribution of unfrozen fraction and salt concentration to the survival of slowly frozen human erythrocytes. *Cryobiology*, **22**(6), 509-536 (1985).

7. Robbins, R. K., Saacke, R. and Chandler, P. T. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. *Journal of Animal Science,* **42**(1), 145-154(1976).

8. Akhter, S., Ansari, M. S., Andrabi, S. M. H., Ullah, N. and Qayyum, M. Effect of antibiotics in extender on bacterial and spermatozoal quality of cooled buffalo (Bubalus bubalis) bull semen. *Reproduction in Domestic Animals*, **43**(3), 272-278(2008).

9. Senger, P. L. Handling frozen bovine semen-Factors which influence viability and fertility. *Theriogenology,* **13**(1), 51-62(1980).

10. Holt, W. V. Basic aspects of frozen storage of semen. *Animal Reproduction Science,* **62** (1-3), 3-22(2000).

11. Salisbury, G. W. and VanDemark, N. L. Physiology of reproduction and artificial insemination of cattle. Physiology of reproduction and artificial insemination of cattle (1961) Publisher : W.H. Freeman & Company; First Edition (January 1, 1961).

12. Vishwanath, R., and Shannon, P. Storage of bovine semen in liquid and frozen state. *Animal Reproduction Science*, **62**(1-3), 23-53(2000).

13. Robbins, R. K., Oconnor, M., Chandler, P. T. and Saacke, R. G. Freezing of bovine semen in French straws. Journal of Animal Science, 37(1), 327-327(1973).

14. Rodriguez, O. L., Berndtson, W. E., Ennen, B. D. and Pickett, B. W. Effect of rates of freezing, thawing and level of glycerol on the survival of bovine spermatozoa in straws. *Journal of Animal Science*, **41**(1), 129-136(1975).

15. Eriksson, B. M., and Rodriguez-Martinez, H. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. *Animal Reproduction Science,* **63**(3-4), 205-220(2000).

16. Söderquist, L., Madrid-Bury, N. and Rodriguez-Martinez, H. Assessment of ram sperm membrane integrity following different thawing procedures. *Theriogenology,* **48**(7), 1115-1125(1997).

17. Gordon, I., 2002. Controlled Reproduction in Cattle and Buffaloes. CABI Publishing, Wallingford, UK.

18. Mitchell, J. R. and Doak, G. A. The Artificial Insemination and Embryo Transfer of Dairy and Beef Cattle (Including Information Pertaining to Goat, Sheep, Horses, Swine and Other Animals): a handbook and Laboratory Manual. 9th Edn., Upper Saddle River, Pearson Education Inc., NJ, pp: 57-135 (2004).

19. Bravo, P. W., Skidmore, J. A. and Zhao, X. X. Reproductive aspects and storage of semen in Camelidae. *Animal Reproduction Science,* **62**(1-3), 173-193(2000).

20. Calamera, J., Buffone, M., Doncel, G., Brugo-Olmedo, S., de Vincentiis, S., Calamera, M., Storey, B. and Alvarez, J., Effect of thawing temperature on the motility recovery of cryopreserved human spermatozoa. *Fertility and Sterility*, **93**(3), 789-794(2010).

21. Lionel Wildy, M. Comparison between Sperm Preparation before Cryopreservation and Post-Thawing Sperm Preparation using Density Gradient Centrifugation in Normozoospermic Semen Samples. *Journal of Infertility and Reproductive Biology,* **10**(2), 19-21(2022). https://doi.org/10.47277/JIRB/10(2)/19.

22. Lionel Wildy, M., Boyd, L., Fourie, J., Makoyo, O. and Huyser, C. The Development of a Simplified Swim-up Method for Sperm Processing. *Journal of Infertility and Reproductive Biology,* **9**(4),160-167(2022).https://doi.org/10.47277/JIRB/9(4)/160.

23. Kumar, N. and Singh, A. K. Semen Quality Parameters in Male Partners of Infertile Couples and Their Correlation with Socio-demographic Features in Rural Tertiary Care Center of Southern India: An Observational Study. *Journal of Infertility and Reproductive Biology,* **10**(1), 1-6(2022).

24. Aderemi, A. S., Samson, O. B., Akomaye, A. J. and Gabriel, I. A. Spermatogenic and steroidogenic functions of rat testis following exposure to Alafia barteri leaf extracts. *Journal of Infertility and Reproductive Biology*, **7**(2), 11-16(2019).

25. Emokpae, M. A., and Olaode, O. Dose-Dependent Effect of Garlic (*Allium sativum*) Extract Feeding on Semen Characteristics and Testes in Wistar Rats. *Journal of Infertility and Reproductive Biology,* **9**(3), 130-135(2021).

26. Ojatula, A. O. Spermatogenic Efficacy of *Pausinystalia yohimbe* (K. Schum.) Pierre ex Beille Roots in Male Rats. *Journal of Infertility and Reproductive Biology*, **8**(2), 9-17 (2020).