Indian Journal of Animal Sciences **92** (1): 55–58, January 2022/Short communication
https://doi.org/10.56093/ijans.v92i1.120918

Semen alkaline phosphatase activity reveals a lack of association with bull semen freezability

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Received: 22 February 2021; Accepted: 26 October 2021

Keywords: Alkaline phosphatase activity, Biochemical marker, Cryopreservation, Frieswal bulls, Semen

A large number of research studies have been conducted on the structural, functional, and molecular changes of sperms during the freezing-thawing process to identify freezability biomarkers (Ryu *et al.* 2019, Peris-frau *et al.* 2020). As the underlying mechanisms of genetic differences are unknown, identification of freezability markers might be the most efficient approach for improving semen cryopreservation (Fraser 2017). The rich biochemical components of seminal plasma could serve as important biomarkers for infertility (Feng *et al*. 2015) and freezability (Juyena and Stelletta 2012). It is vital to identify the ejaculate with good freezability by determining the biochemical makeup of the ejaculate at the pre-freeze stage (Juyena and Stelletta 2012).

Alkaline Phosphatase (ALP) is one of the important seminal enzymes (Gupta *et al.* 2019). Its activity plays an essential role in sperm motility by phosphorylating the proteins by cAMP-dependent protein kinase enzyme action (Juyena and Stelletta 2012). Its activity symbolizes the health status of accessory glands, sperm metabolism, and integrity of the plasma membrane (Gupta *et al.* 2019). Multiple studies indicate the use of measuring ALP activity for assessing various seminal parameters and sperm functions in different species such as duct blockages in equine (Turner and McDonnell 2003), true ejaculate identification in rhinos (Roth *et al.* 2010) and dogs (Memon 2007), *in vitro* capacitated sperm detection in boar (Bucci *et al.* 2014). However, the studies on the use of this enzyme as a freezablility biomarker are limited. Considering the importance of this enzyme in semen biology, fertility (Turner and McDonnell 2003), sperm motility (Juyena and Stelletta 2012), etc. the present study assessed its use as a biochemical marker for freezability at the pre-freeze stage. It could benefit the cattle industry via the early elimination of low freezable semen and bulls.

A total of 450 ejaculates from 22 randomly selected Frieswal (5/8 HF × 3/8 Sahiwal) bulls of 3–6 years of age, maintained under identical management at the Central Institute for Research on Cattle, India, were evaluated in this study. All the procedures followed after the ethical approval of the Institute Animal Ethical Committee (IAEC) as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. An average of 22 ejaculates, ranging from 10 and 33, per bull, were recorded for seminal parameters, viz. initial motility, concentration, volume, and post-thaw motility using standard semen procedures of the laboratory. A total of 14 bulls recorded with similar initial motility were grouped into good and poor freeze-groups based on their post thaw motility (PTM) %. Consecutive ejaculates (3 ejaculates in four days) from Good (*N*=6) and Poor (*N*=8) freeze-groups bulls were collected and fractionated to seminal plasma and sperm by centrifugation at 350 g for 30 min at ambient temperature. The sperm fractions were washed twice in phosphate-buffered saline (PBS) and stored at –80°C in PBS until further analysis. A total of 50×10^6 sperm cells were lysed in TritonX-100 buffer for 1 h (Alyethodi *et al.* 2020), and the lysates were collected by centrifugation at 10,000 rpm for 10 min. Total sperm lysates from Good (*N*=6) and Poor (*N*=8) and seminal plasma Good (*N*=14) and Poor (*N*=19) were utilized for present assessment. The seminal plasma fractions (20 µl) and sperm lysates (80 µl) were processed to estimate alkaline phosphatase enzyme assay as per the manufacturer's instructions (EZAssay^{TM} Alkaline Phosphatase Activity Estimation Kit, Himedia, India).The absorbance was measured in 96-well transparent polypropylene microplate using a Alere Microplate Reader (Alere Medical Pvt Ltd, India, AM 2100) at 405 nm. The level of the enzyme was expressed in units/ml.

An independent sample t-test was performed to evaluate the effect of freeze-groups on alkaline phosphatase activity. Kinetics of alkaline phosphatase in consecutive semen collections were estimated using two-tailed paired t-tests against the first day estimates. The correlation of alkaline phosphatase activity with semen volume and sperm concentration was assessed by Pearson correlations. Data were presented as least squared means (± SEM), and values

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were considered statistically significant when the p-value was <0.05.

The present study recorded an average ALP activity of 16.00 U/ml of seminal plasma. It ranged between 8.68–20.78 U/ml of seminal plasma. The 50×10^6 spermatozoa yielded an average ALP activity of 2.35 U/ml of sperm lysate, which varied from 0.02 to 4.1 U/ml. The ALP activity in sperm and seminal plasma of both freeze-groups showed no significant difference (Table 1). Similarly, the alkaline phosphatase activity showed no correlation with the semen volume or sperm concentration. Semen, when collected on consecutive days, did not alter its ALP activity (Fig. 1).

The mean seminal plasma ALP activity was higher than the previous report (Reid *et al.* 1948), where authors reported an average of 3.93 U/ml with a range of 0.97 to 34.59 U/ml. However, the estimated level of sperm ALP is in agreement with the previous report (Pero *et al.* 2017). In general, the semen ALP activities reported by various authors are often inconsistent. It might be because the ALP activity is influenced by the ration (Reid *et al.* 1948), season (Knecht *et al*. 2014), and age (Alexander *et al.* 1971) of the animal. In stallion, it is reported that ALP have reduced activity upon freezing. Also, the *p*H of the semen extender influences its activity (Bucci *et al.* 2016).

The present study results indicate a lack of correlation between ALP activity with sperm concentration and semen volume. It is in accordance with the previous reports in bull (Reid *et al.* 1948), ram (Asadpour 2012), and human (Jitendra *et al*. 2015). Contrarily, ALP activity positively correlates with sperm concentration in rhinos, buffalos, and boars (Roth *et al.* 2010, Rodríguez *et al.* 2013, Viviane *et al*. 2020) and negatively correlates in stallion (Talluri *et al.* 2017). The ALP activity reported negatively correlated with the semen volume in boars (Rodríguez *et al.* 2013) and stallions (Talluri *et al.* 2017). These findings indicate that the actual role of ALP depends on multiple factors and varies with species.

Studies on the role of ALP activity in semen cryopreservation are scarce in the literature. The present study's findings indicate that ALP activity may not be a reliable biomarker for semen cryopreservation. Previously, it was reported that the ALP activity of frozen/thawed bull semen has no role in fertility parameters (Pero *et al.* 2017). Contrarily, higher ALP and Alanine transaminase activities, measured at different stages of bull semen processing (fresh diluted, equilibration, 0 h post-thaw, and 1 h post-thaw), are reported in the semen of good freezable group (Gupta *et al.* 2019). The authors reported significant differences in mass motility between the studied good and poor freeze-groups (Gupta *et al.* 2019). In the present study, all the bulls inducted in both groups had similar initial motility. Further, aminimum standard parameters of semen volume and sperm concentrations are ensured to minimize the confounding effects of these seminal parameters, if it exists, on alkaline phosphatase activity. Hence, this study points out that ALP activity may not play a significant role in bull semen's cryopreservation.

Table 1. Alkaline phosphatase (ALP) activity in spermatozoa and seminal plasma of bulls

Category	Freeze-groups	N	Alkaline phosphatase (U/ml)*	Range (U/ml)
Spermatozoa	Good	6	2.04± 0.91	0.02–3.3
	Poor	8	2.67±0.65	0.59–4.1
Seminal plasma	Good	14	15.42±1.05	8.65–20.28
	Poor	19	16.59±0.88	11.00–20.78

*Data presented as least square means value (LSM±SE).

Leakage of multiple enzymes such as ALP, glutamic oxaloacetic (GOT), glutamic pyruvic transaminase (GPT), acid phosphatase (ACP) andlactic dehydrogenase (LDH) in post-thaw semen arereasoned as responsible for poor freezability in buffalo (Dhami and Kodagali 1990). In ram, ALP leakage in seminal plasma is proposed as a marker for optimizing cooling and freeze-thaw steps of cryo-preservation (Upreti *et al.* 1996). The designs of these experiments might indicate that leakage of ALP is one of the reasons for the harmful effects of freezability and associated sperm changes. The present study evaluated ALP activity at the neat semen stage as a marker for predicting the freezability of the semen. With the design of this experiment, the present study findings indicate that the ALP estimates at the pre-freeze stage may not indicate bull semen freezability.

The present study detected no significant variation in consecutive ejaculates collected at short intervals in the ALP enzyme activity. We have previously reported a lack of adverse effects of repeated semen collection at short intervals on semen volume and concentration (Alyethodi *et al.* 2021). The same study showed that the total antioxidant capacity is improved, and lipid peroxidation is decreased when multiple ejaculates are collected at short intervals. The present study gives partial insight that collecting consecutive ejaculates with a short abstinence period may not influence the seminal ALP activity.

Alkaline phosphatase (ALP) is one of the important seminal enzymes and plays an essential role in sperm motility. The ALP activity in seminal plasma seems species-specific. The present study's findings indicate that ALP activity may not play a significant role in bull semen's cryopreservation. Its activity did not correlate with the

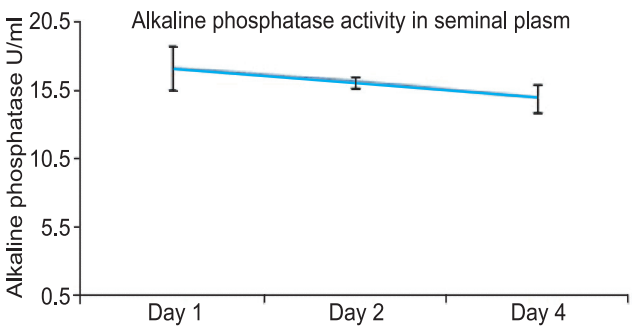

Fig. 1. Kinetics of Alkaline phosphatase activity.

sperm concentration and semen volume. Also, collecting consecutive ejaculates with a short abstinence period may not influence the seminal ALP activity. The present study findings indicate that the sperm and seminal plasma's ALP activity levels in bulls may not be a reliable indicator of semen freezability.

SUMMARY

The present study aimed to measure and compare alkaline phosphatase (ALP) enzyme activity in the bull's semen with varying freezability. Based on the post-thaw motility percentages, the bulls of similar age groups were grouped into good and low freezable semen groups (Freeze-groups). A total of 450 ejaculates from 21 bulls, with an average of 22 ejaculates per bull, were initially screened. Bulls (14) with similar initial motility and qualifying minimum standard semen volume and sperm concentration values were inducted in the final study. Consecutively two to three ejaculates collected from each bull were processed for alkaline phosphatase activity. Association of alkaline phosphatase activity with freezability was carried out using an independent t-test. Correlations of alkaline phosphatase with semen volume and sperm concentrations were assessed using Pearson correlations. Alkaline phosphatase activity showed an insignificant variation between freeze-groups. Further, no correlation of enzyme activity with the semen volume and sperm concentration was observed. Besides, seminal plasma alkaline phosphatase activity assessed on consecutive days of ejaculates showed no variation. The preliminary finding of this study indicates that alkaline phosphatase may not be involved in bull semen freezability. Hence, it may not be a reliable biochemical marker or indicator in bull cryopreservability studies.

ACKNOWLEDGEMENTS

The authors are thankful to the Departmentof Science and Technology (DST)-Science and Engineering Research Board (SERB) for funding this project. We are grateful to the Director, ICAR-CIARI, Port Blair, and the Director, ICAR-CIRC, Meerut for providing necessary facilities to carry out the present research. We are also thankful to Military Farm, Meerut, India, for giving experimental animals. We express our gratitude to HoD, AGB and Dr Sushil Kumar for their kind support in sample collection.

REFERENCES

Alexander F C M, Zemjanis R, Graham E F and Schmehl M L. 1971. Semen characteristics and chemistry from bulls before and after seminal vesiculectomy and after vasectomy. *Journal of Dairy Science* **54**: 1530–35.

Alyethodi R R, Karthik S, Muniswamy K, Ravi S K, Perumal P, Bhattacharya D, Bala P A, De A K, Sujatha T, Sunder J and Kundu A. 2020. Assessment of protein profiles of RNA later stored and fresh PBMC cells using different protein extraction buffers. *Protein Journal* **39**(3): 291–300.

Alyethodi R R, Sirohi A S, Karthik S, Tyagi S, Perumal P, Singh U, Sharma A and Kundu A. 2021. Role of seminal MDA, ROS, and antioxidants in cryopreservation and their kinetics under the influence of ejaculatory abstinence in bovine semen. *Cryobiology* **98**: 187–93.

Asadpour R. 2012. Relationship between mineral composition of seminal plasma and semen quality in various ram breeds. *Acta Scientiae Veterinariae* **40**.

Bucci D, Giaretta E, Spinaci M, Rizzato G, Isani G, Mislei B, Mari G, Tamanini C and Galeati G. 2016. Characterization of alkaline phosphatase activity in seminal plasma and in fresh and frozen–thawed stallion spermatozoa. *Theriogenology* **85**: 288–95.

Bucci D, Isani G, Giaretta E, Spinaci M, Tamanini C, Ferlizza E and Galeati G. 2014. Alkaline phosphatase in boar sperm function. *Andrology* **2**: 100–06.

Dhami A J and Kodagali S B. 1990. Freezability, enzyme leakage and fertility of buffalo spermatozoa in relation to the quality of semen ejaculates and extenders. *Theriogenology* **34**: 853–63.

Feng R, Lu J, Zhang H and Lü N. 2015. A Pilot Comparative Study of 26 Biochemical Markers in Seminal Plasma and Serum in Infertile Men. *Biomed Research Interantional*.

Gafer, Jehan El Rahman, Ghada and Rawash M. 2015. Association of *Hsp70* gene polymorphism and bull semen quality in winter and summer seasons. *Alexandria Journal of Veterinary Sciences* **46**: 1.

Gupta S, Singh M, Dhaka Y and Kumar P. 2019. Functional and biochemical attributes of bull semen with reference to freezability at different stages of semen processing. *Harayana Veterinarain* **58**: 73–77.

Jitendra N, Piyush P, Sangeeta P and Bipin C. Study of seminal acid phosphatase and alkaline phosphatase level in relation to sperm count in teaching hospital. *National Journal of Medical Research* **4**.

Juyena N S and Stelletta C. 2012. Seminal plasma/ : An essential attribute to spermatozoa. *Journal of Andrology* **33**: 536–51.

Knecht D, Œrodoñ S and Duziński K. 2014. The influence of boar breed and season on semen parameters. *South African Journal of Animal Science* **44**: 1–9.

Leyland Fraser. 2017. Markers for Sperm Freezability and Relevance of Transcriptome Studies in Semen Cryo-preservation: A Review. *Theriogenology*, Rita Payan Carreira, IntechOpen, DOI: 10.5772/intechopen.68651.

McCue P M. 2014. Alkaline phosphatase: A marker for ejaculation. *Equine Reproductive Procedures.* 401–02.

Memon M A. 2007. Common causes of male dog infertility. *Theriogenology* **68**: 322–28.

Peris-frau P, Soler A J, Iniesta-cuerda M, Mart A, Irene S, Alejandra D, Garc O, Maroto-morales A, Montoro V and Juli J. 2020. Sperm cryodamage in ruminants: Understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *International Journal of Molecular Medicine* **21**(8): 2781.

Pero M E, Lombardi P, Longobardi V, Boccia L, Vassalotti G, Zicarelli L, Ciani F and Gasparrini B. 2017. Influence of γ-glutamyl transferase and alkaline phosphatase activity on in vitro fertilisation of bovine frozen/thawed semen. *Italian Journal of Animal Science* **16**: 390–92.

Reid J T, Ward G M and Salsbury R L. 1948. Acid and alkaline phosphatase levels in consecutive semen ejaculates from bulls. *The American Journal of Physiology* **153**: 235–41.

Rodríguez A L, Rijsselaere T, Beek J, Vyt P, Van Soom A and Maes D. 2013. Boar seminal plasma components and their relation with semen quality. *Systems Biology in Reproductive*

Medicine **59**: 5–12.

Roth T L, Stoops M A, Robeck T R, Ball R L, Wolfe B A, Finnegan M Vand O'Brien J K. 2010. Alkaline phosphatase as an indicator of true ejaculation in the rhinoceros. *Theriogenology* **74**: 1701–06.

Ryu D Y, Song W H, Pang W K, Yoon S J, Rahman M S and Pang M G. 2019. Freezability biomarkers in bull epididymal spermatozoa. *Scientific Reports* **9**: 1–9.

Talluri T R, Mal G and Ravi S K. 2017. Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. *Veterinary World* **10**: 214–20.

Turner R M O and McDonnell S M. 2003. Alkaline phosphatase in stallion semen: Characterization and clinical applications. *Theriogenology* **60**: 1–10.

Upreti G C, Payne S R, Duganzich D M, Oliver J E and Smith J F. 1996. Enzyme leakage during cryopreservation of ram spermatozoa. *Animal Reproduction Science* **41**: 27–36.

Viviane A, Codognoto M, Yamada P H and Salgado S. 2020. Correlation between alkaline phosphatase of seminal plasma and sperm parameters in buffalos. *Revista CES Medicina Veterinaria y Zootecnia* 10–11.