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# EFFECT OF VITAMIN C AND E SUPPLEMENTATION TO TRIS EGG YOLK EXTENDER ON CANINE EPIDIDYMAL SPERMATOZOA FOLLOWING CRYOPRESERVATION

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## ABSTRACT

Cryopreservation of spermatozoa is a method of choice to preserve genetic potential of fertile proven dogs when they die unexpectedly or fail to ejaculate. In this scenario, present study was designed to monitor the individual and combined effects of supplementation of two antioxidants (Vitamin E 0.3mM and Vitamin C 0.90mg) to freezing extender tris egg yolk (TEY) via cryopreservation on the canine epididymal sperm quality parameters. For this purpose, testes were collected from 20 healthy dogs and cauda epididymal semen samples were extracted after dissection. The collected samples were evaluated for sperm motility and viability after that extended in TEY extender. The extended semen samples were loaded in 0.25ml straws and plunged into liquid nitrogen after holding the straws in liquid nitrogen vapors for two minutes then plunged into $LN_2$. Then the frozen samples were thawed for 15 seconds at 50°C and subjected to post-thaw evaluation through CASA. The data were analyzed using one-way ANOVA under CRD and means were compared using Tukey's test. Results revealed that the post-thaw semen parameters progressive sperm motility, viability, plasma membrane integrity and motion kinetics straight-line velocity, curvilinear velocity increased significantly ($P< 0.05$) in the TEY with vitamin C and vitamin E groups, except for amplitude of lateral head displacement which was significantly decreased ($P< 0.05$), compared to control group. Based on the results of this study, it was concluded that supplementation of vitamin C and E alone or in combination with TEY extender significantly ($P< 0.05$) improved canine epididymal sperm quality parameters following cryopreservation.

**Keywords:** cryopreservation, epididymal spermatozoa, motility, tris egg yolk extender, vitamin C and E

## INTRODUCTION

Commercial dog breeding has gained popularity in recent years, with a focus on improving genetics and reproducing pups with high zootechnical and economic values (Jang *et al.*, 2010). Domestic dogs provide great experimental models because of their reproductive physiology, which is similar to that of wild animals and humans (Nagashima and Songsasen, 2021). Cryopreservation of spermatozoa is a useful method for maintaining genetic diversity and preserving genetic material in endangered wild species, including canids particularly in males with obstructive azoospermia and oligospermia (Sicherle *et al.*, 2020).

One typical practice for assisted reproduction is to extract semen straight from the vas deferens and epididymal tails (Ambar *et al.*, 2021). Sperm viability may be compromised by cryopreservation, which can harm the sperm membrane irreversibly. The formation of intracellular ice crystals or osmotic shock during freezing and thawing, thermal shock during semen cooling, stress, and the action of cryoprotectants are some of the stressors that sperm cells encounter throughout the cryopreservation process. Epididymal spermatozoa are vulnerable to oxidative imbalance due to intra and extra-cellular changes during maturation, including the loss of cytoplasm and an increase in polyunsaturated fatty acid proportion in the plasma membrane (Angrimani *et al.*, 2014). Furthermore, lipid peroxidation products such malondialdehyde

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(MDA) might be as damaging as ROS, causing DNA breakdown and decreased reproduction potential (Kawai *et al.*, 2017).

Poor sperm quality can result from nutrient deficiencies that affect spermatogenesis or result in extreme oxidative stress. Oxidative modifications lead to sperm dysfunctions such decreased motility and viability while also sperm-oocyte fusion due to the production of ROS. The addition of different antioxidants to freezing extenders improves the quality of the semen after thawing in order to mitigate the detrimental effects of ROS. The most crucial defenses against oxidative stress brought on by free radicals are antioxidants (Silva *et al.*, 2011). Antioxidants may therefore be crucial in protecting male germ cells from oxidative harm. A potent antioxidant in the human body is vitamin E (α-tocopherol) and it is a well-known antioxidant that prevents sperm cell membrane damage caused by free radicals and is essential for shielding spermatozoa from lipid peroxidation (Qazi *et al.*, 2019). Antioxidants come naturally in vitamins E and C. Since vitamin E is soluble in lipids, it may act as the first line of defense against polyunsaturated fatty acid peroxidation on membrane phospholipids. Furthermore, vitamin E prevents ROS from damaging the cell membrane, neutralizes free radicals, and improves how well other antioxidants work. Water-soluble antioxidant vitamin C serves as a crucial cofactor in the hydroxylation and amination reactions. The creation of collagen, proteoglycan, and other elements of the intercellular matrix involves ascorbic acid and vitamin E (Priyanto *et al.*, 2023).

Because compared to ejaculated spermatozoa, epididymal spermatozoa are more vulnerable to ROS. Investigating if ascorbic acid may shield epididymal spermatozoa from cryoinjury is worthwhile. Thus, the current study sought to examine how vitamin C and E affected the motility, viability, plasma membrane integrity, and morphological abnormalities of canine epididymal spermatozoa during cryopreservation and thawing.

## MATERIALS AND METHODS

The study was carried out at the Department of Theriogenology, University of Agriculture Faisalabad, Punjab Pakistan.

### Collection and processing of testes

After a routine orchidectomy at local pet clinics, testes were collected within 1 hour from 20 healthy dogs of various breeds. The testes with epididymides were wrapped in polythene bubble paper and immediately kept in a Styrofoam box at refrigeration temperature (4ºC) and transported to the lab within 2 hours. The testes were brought to room temperature in the lab and left there for 20 minutes. Under aseptic conditions each dog caudal epididymides were separated and chopped into small pieces using a scalpel and kept in a separate petri plate with saline (Fayez *et al.*, 2021).

### Initial semen evaluation

Macro and microscopic examination was carried out for the samples of each dog semen separately as they were not pooled. A phase contrast microscope used to evaluate sperm motility and morphology. Semen samples exhibiting >70% motility and 70% normal morphology were processed further.

### Preparation of Semen extenders

Tris egg yolk (TEY) (Tris 2.42g, citric acid 1.34g, fructose 1g, egg yolk 20% v/v, glycerol 7% v/v, benzyl penicillin 1000 IU/ml, and streptomycin sulphate 1000 mg/ml, double distilled water 73ml to make final volume 100ml extender with pH 6.9) were used for the extension of epididymal semen. Chemicals for the preparation of TEY extender were procured from Sigma-Aldrich Chemicals Co., USA**.**

### Semen dilution

Four treatment groups were formed supplemented with two antioxidants vitamin C (Ascorbic acid) 0.90mg and vitamin E (α-tocopherol) 0.3mM and divided into groups as A (Control group having TEY), $A_1$ (TEY + Vit C 0.90mg), $A_2$ (TEY + Vit E 0.3mM) and $A_3$ (TEY+ Vit C 0.90mg + Vit E 0.3mM). At room temperature (22°C), extenders were added to epididymal sperms to achieve the final sperm concentration to $50x10^6$/ml.

### Processing of semen

The semen was cooled to 4ºC within 2 hours by placing the semen in a falcon tube at a cooling rate of 0.3ºC/minute. Extended samples of semen were filled into 0.25ml pre-labeled straws at 4ºC, to provide a final concentration of $50x10^6$ sperms/straw. After filling straws were maintained at 4ºC for 2 hours (Netherton *et al.*, 2022) then placed 3cm above the liquid nitrogen surface in a Styrofoam box for 2 minutes then plunged into $LN_2$.

### Post-thaw evaluation of semen

For post-thaw assessment, two straws per extender were thawed in a water bath at 50°C for 15 seconds. Sperm were assessed using a computer-assisted sperm analyzer (CASA). On a glass slide that had been preheated and equipped with a stage heating system that was kept at 37°C, a 7µl sample of semen was put. Post thaw sperm motility, viability, plasma membrane integrity, morphological abnormalities and motion kinetics were recorded. The integrity of the sperm cell plasma membrane was evaluated using the Hypo-Osmotic Swelling Test (Lodhi *et al.*, 2008) and the motion dynamics were recorded using CASA, including the amplitude of lateral head displacement (ALH) µm, beat cross-frequency (BCF) Hz, average path velocity (VAP) µm/s, straight line velocity (VSL) µm/s, and curvilinear velocity (VCL) µm/s (Zmudzinska *et al.*, 2022).

### Statistical analysis

Collected data were analyzed using one-way ANOVA under CRD and Tukey's test for comparing the means (Suharyati *et al.*, 2024).

## RESULTS

Present research showed that post-thaw progressive motility (%), viability (%), plasma membrane integrity (%) were significantly (P-value< 0.05) improved by supplementing vitamin C or E than control group. The effect of adding vitamin C and E to dog epididymal semen diluted with TEY on sperm characteristics following cryopreservation are presented in (Table 1). Morphological abnormalities were also significantly reduced by supplementing vitamin C and E. The rest of the sperm motion kinetics including post-thaw straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) were highest at supplementation of both vitamin C and E along with tris extender. Table 2 showed statistically significant improvement following vitamin C and E supplementation.

## DISCUSSION

The dog epididymal spermatozoa was cryopreserved using the common cooling procedure in this investigation. Until now, little or no research has been conducted on this approach. When their bitches go into heat, dog breeders confront a difficulty; nevertheless, thanks to this stored sperm, it can now impregnate the bitch with suitable sperm at the right time. The main objectives of this research were to determine the influence of TEY extender addition with vitamin C and E on the efficiency of canine epididymal sperm cryopreservation. For dogs those are unable to ejaculate, require castration as a kind of rehabilitation, or pass away tragically, canine epididymal semen offers an extra source of gametes to preserve the genetics of key breeding dogs. Additionally, as neutering of non-breeding dogs is rather common, creating a gene reserve of epididymal sperm collected following castration would significantly help to expand the genetic variation of dogs (Ali Hassan *et al.*, 2021).

In the present study epididymal spermatozoa was collected through mincing technique by adding physiological saline and initially analyzed for motility and viability. The mean ± SEM values before extension for motility were 71.470 ± 2.532 and viability 78.22 ± 4.75. (Prapaiwan *et al.*, 2016) found a similar result values after the initial evaluation of epididymal spermatozoa. (Hori *et al.*, 2015) also found similar results while checking the influence of different methods of collection from the canine epididymides. Techniques for harvesting epididymal sperm include epididymal mincing, in vitro epididymal sperm aspiration after epididymectomy, and in vivo percutaneous epididymal sperm aspiration. According to Hassan *et al.* (2021) the float-up method, also known as epididymal mincing, is a fantastic one-time collecting technique that may be employed after a post-mortem or orchiectomy. (Chima *et al.*, 2017) also conducted a study in local dogs to evaluate the cauda epididymal sperm parameters recovered post-castration.

**Table 1.** Mean ± SEM values for the post-thaw progressive motility (%), viability (%), plasma membrane integrity (%) and morphological abnormalities of spermatozoa

| Treatments | Progressive motility (%) | Viability (%) | Plasma membrane integrity (%) | Morphological abnormalities (%) |
|---|---|---|---|---|
| A | $23.57\pm2.95^{C}$ | $27.87\pm2.25^{C}$ | $20.39\pm1.70^{C}$ | $20.85\pm1.41^{A}$ |
| $A_1$ | $28.63\pm0.71^{B}$ | $34.06\pm2.33^{B}$ | $27.09\pm2.05^{B}$ | $19.93\pm1.84^{A}$ |
| $A_2$ | $30.65\pm0.77^{B}$ | $36.36\pm2.58^{B}$ | $29.18\pm4.1^{B}$ | $18.07\pm0.93^{B}$ |
| $A_3$ | $35.87\pm3.01^{A}$ | $41.46\pm2.42^{A}$ | $36.54\pm2.51^{A}$ | $17.6\pm0.74^{B}$ |

Means with different superscripts as A, B is different significantly ($P< 0.05$)

**Table 2.** Mean ± SEM values for the post-thaw VSL, VCL, VAP, ALH and BCF of spermatozoa

| Treatments | VSL (µm/s) | VCL (µm/s) | VAP (µm/s) | ALH (µm) | BCF (Hz) |
|---|---|---|---|---|---|
| A | 9.25± 0.71$^{C}$ | 21.79± 1.77$^{B}$ | 10.77± 1.21$^{C}$ | 0.91± 0.34$^{A}$ | 4.05± 0.291$^{A}$ |
| $A_1$ | 11.31± 1.07$^{B}$ | 24.67± 2.31$^{B}$ | 14.70± 1.12$^{B}$ | 0.74± 0.09$^{AB}$ | 4.051± 0.22$^{A}$ |
| $A_2$ | 12.58± 0.79$^{A}$ | 28.88± 2.08$^{A}$ | 15.31± 1.5$^{AB}$ | 0.74± 0.15$^{AB}$ | 4.18± 0.17$^{A}$ |
| $A_3$ | 13.37± 1.18$^{A}$ | 30.65± 3.89$^{A}$ | 17.12± 2.79$^{A}$ | 0.61± 0.14$^{B}$ | 4.20± 0.19$^{A}$ |

Means with different superscripts as A, B is different significantly ($P<$ 0.05)

In present study the evaluation of epididymal spermatozoa after extension with the TEY extender, the mean ± SEM values were noticeably greater for motility, viability, and plasma membrane integrity ($P<$ 0.05) while morphological sperm abnormality was significantly lower after extension with the TEY extender. Prapaiwan *et al*. (2016) found a similar result values after the initial evaluation of epididymal spermatozoa after extension with Tris buffer extender. Kurien *et al.* (2013) compared the impact of rapid freezing and three distinct semen extenders on the post-thaw survivability of dog semen and their study indicated that the Tris extender was superior to Triladyl® in fast freezing, these studies agree with the present research. (Abdul Rauf *et al.*, 2022) conducted an experiment to evaluate the effects of ascorbic acid (2.1mg/mL). Tris-citric acid-yolk (TCY) extender was fortified with ascorbic acid, and it was compared to Triladyl extender for preserving bovine semen by cryopreservation.

In the present study the mean ± SEM value showed that the addition vitamin C 0.90mg and E 0.3mM had significant effect ($P<$ 0.05) on the post-thaw progressive motility of canine epididymal spermatozoa. The mean ±S EM value of Group $A_3$ was significantly higher than all the treatment and control groups while group $A_2$ and $A_1$ were non-significantly different ($P>$ 0.05) from each other. Michael *et al.* (2007) undertook a study to assess the quality of frozen dog sperm that had been treated with vitamin C-containing diluents (1.5mM, 6mM) and E (0.3mM, 1.2mM) after being frozen. There was significant ($P<$ 0.001) increase in the post-thaw total motility. Sandeep *et al.* (2015) reported that inclusion of vitamin C (2.5mM) in the extender for diluting the semen had positive impact in post thaw quality of buffalo bull semen. Mittal *et al.* (2014) shown that vitamin C and E supplements (5 mM + 5mM) for extenders improved the motility of cryopreserved spermatozoa of bulls, these studies agree with the present research. Similar findings were in boar spermatozoa post thaw motility by the addition of vitamins E and C simultaneously (Ghiuru *et al.*, 2010). The results of Martínez-Páramo *et al.* (2012) demonstrated that extenders supplemented with α-tocopherol (0.1mM) or ascorbic acid (0.1mM) improved sea bass sperm motility. Franco *et al.* (2013) supplemented the varying amounts of ascorbic acid (0.4, 0.9 and 1.8g/L) and α-tocopherol (0.5, 1 and 2mM) in extender. The supplementation of α-tocopherol improved the post thaw motility and their results were like present study but the higher concentration of ascorbic acid had a negative effect on post thaw motility which is contrary to present study.

Franco *et al.* (2013) studied the addition of α-tocopherol (0.5, 1 and 2mM) and ascorbic acid (0.4, 0.9 and 1.8g/L) to extender improved the stability and integrity of the membrane of equine spermatozoa which is similar to present study. Michael *et al.* (2007) conducted a study to assess the viability of frozen dog semen that has undergone vitamin C (1.5mM, 6mM) and E (0.3mM, 1.2mM) processing after thawing. There was significant ($P<$ 0.001) increase in the post-thaw livability in vitamin E group, the study agrees with the present research, but vitamin C had showed no effect on post-thaw viability which is contrary to present study.

Mittal *et al.* (2014) reported bull spermatozoa that had been cryopreserved benefitted from vitamin C and E (5mM + 5mM) extender addition in terms of motility, livability and plasma membrane integrity. Extender that was combined with vitamins E and C improved the performance of frozen-thawed boar spermatozoa (Izquiedo *et al.*, 2017), these studies agree with the present research. Abdul Rauf *et al.* (2022) reported that the motility and viability of bovine spermatozoa were enhanced by the addition of ascorbic acid (2.1mg/mL) in tris-citric acid-yolk (TCY) extender cryopreservation of semen which is like present study.

In present study the mean ±SEM value showed that the addition vitamin C 0.90 mg and E 0.3mM had significant effect ($P<$ 0.05) on the kinematics parameters of canine epididymal spermatozoa. Martínez-páramo *et al.* (2012) study agrees with the current research in that it

was found that adding α-tocopherol (0.1mM) and ascorbic acid (1mM) to the freezing solution considerably increased the sea bass sperm straight-line velocity (VSL) and curvilinear velocity (VCL). Abdul Rauf *et al.* (2022) concluded that the average path length, curved path length, and straight-line path length were all noticeably higher ($P< 0.05$) in the ascorbic acid (2.1mg/mL) group as compared to the Triladyl group but the average path length, beat cross frequency (BCF) and straight-line length were greater in the Triladyl group as compared to the citric acid-yolk (TCY) group which is contrary to present study.

## CONCLUSION

Result revealed that semen parameters like sperm motility, plasma membrane integrity, livability and CASA motion kinetics like VSL, VCL, etc. increased significantly ($P< 0.05$) in the Tris egg yolk (TEY) with vitamin C 0.90mg and E 0.3mM group except for amplitude of lateral head which was significantly decreased ($P< 0.05$) as compared to control group in which no supplement was added. Based on the results of this research, supplementation of vitamin C 0.90mg and E 0.3mM alone or in combination in both extenders significantly improves the canine epididymal sperm parameters cryopreserved through vitrification technique. It is also observed that the performance of home based TEY Extender is better than commercially available Triladyl® extender in case of canine epididymal spermatozoa.

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## CONFLICT OF INTEREST

With relation to everything connected to this document, we attest that there are no conflicts of interest.

## AUTHOR'S CONTRIBUTION

**M. Waqas:** Contribution as execution of research trial, plagiarism removal and statistical work.

**F. L. Lodhi:** Design the research study.

**R. Kousar:** Write up and assistance in the research.

**S. Umer:** Contributed to execution of research.

**H. Jamil:** Supervised research and manuscript write up.

## REFERENCES

Abdul Rauf, H., R. Qadeer, F. Hassan, M. T. Tunio, M. Bhutta and S. Imran. 2022. Comparative Evaluation of Ascorbic Acid S upplementation in Tris-Egg Yolk Based Extender and Triladyl® Extender on Post-Thaw Kinematics Parameters of Sahiwal Bull Semen. Iranian Journal of Applied Animal Science, 12 (1): 41-48.

Ali Hassan, H., G. Domain, G. C. Luvoni, R. Chaaya, A. Van Soom and E. Wydooghe. 2021. Canine and feline Epididymal seme-a plentiful source of gametes. Animals, 11 (10): 2961.

Ambar, R. F., M. M. Gava, M. Ghirelli-Filho, I. H. Yoshida, T. S. De Paula and S. Glina. 2021. Tissue and sperm handling before assisted reproductive technology (ART): A systematic review. Arab Journal of Urology, 19 (3): 238-246.

Angrimani, D. S. R., C. F. Lucio, G. A. L. Veiga, L. C. G. Silva, F. M. Regazzi, M. Nichi and C. I. Vannucchi. 2014. Sperm maturation in dogs: sperm profile and enzymatic antioxidant status in ejaculated and epididymal spermatozoa. Andrologia, 46 (7): 814-819.

Chima, U. M., A. H. Abu, P. M. Dawuda, A. I. Kisani and T. Ahemen. 2017. Effect of storage time on cauda epididymal sperm parameters of Nigerian local dogs. Open Journal of Veterinary Medicine, 7 (11): 151-161.

Fayez, E., A. Salama, Z. M. Rawash and M. A. El Sayed. 2021. The impact of addition of ascorbic acid to cryopreservation medium on dog epididymal spermatozoa. Veterinary Medical Journal (Giza), 67 (1): 161-173.

Franco, J. S. V., A. Chaveiro, A. Góis and F. M. da Silva. 2013. Effects of α-tocopherol and ascorbic acid on equine semen quality after cryopreservation. Journal of Equine Veterinary Science, 33 (10): 787-793.

Ghiuru, F. V., L. Ioan, I. Roman, A. Hettig, Z. Marius and V. Miclea. 2010. Antioxidant medium for mangalita boar semen cryopreservation. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Anim Sci Biotechnol, 67 (1-2) 445-451.

Hori, T., T. Atago, M. Kobayashi and E. Kawakami. 2015. Influence of different methods of collection from the canine epididymides on post-thaw caudal epididymal sperm quality. Journal of Veterinary Medical Science, 77 (5): 625-630.

Izquiedo, A., A. Iglesias Reyes, R. Cervantes, J. Guerra Liera, J. Insunza Castro, A. Villa Mancera, M. Méndez Mendoza, R. Huerta Crispín, M. de Lourdes Juárez Mosqueda and A. Gómez Vázquez. 2017. Effect of addition of antioxidants in the freezing of boar semen on the motility and viability of sperm. International Journal Current Research, 9: 47599-47600.

Jang, G., M. K. Kim and B. C. Lee. 2010. Current status and applications of somatic cell nuclear transfer in dogs. Theriogenology, 74 (8): 1311-1320.

Kawai, G. K. V., J. R. C. Gurgel, J. D. de Agostini Losano, A. Dalmazzo, C. C. Rocha, R. H. Tsunoda, P. A. de Araujo Goes, B. R. Rui, D. D. S. R. Angrimani and C. M. Mendes. 2017. Susceptibility of stallion spermatozoa to different oxidative challenges: role of seminal plasma. Journal of Equine Veterinary Science, 55: 76-83.

Lodhi, L., M. Zubair, Z. Qureshi, I. Ahmad and H. Jamil. 2008. Correlation between hypo-osmotic swelling test and various conventional semen evaluation parameters in fresh Nili-Ravi buffalo and Sahiwal cow bull semen. Pakistan Veterinary Journal, 28 (4): 186-188.

Martínez-Páramo, S., P. Diogo, M. Dinis, M. Herráez, C. Sarasquete and E. Cabrita. 2012. Incorporation of ascorbic acid and α-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. Theriogenology, 77 (6): 1129-1136.

Michael, A., C. Alexopoulos, E. Pontiki, D. Hadjipavlou-Litina, P. Saratsis and C. Boscos. 2007. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. Theriogenology, 68 (2): 204-212.

Mittal, P. K., M. Anand, A. Madan, S. Yadav and J. Kumar. 2014. Antioxidative capacity of vitamin E, vitamin C and their combination in cryopreserved Bhadavari bull semen. Veterinary World, Veterinary World. 7 (12): 1127-1131.

Nagashima, J. B. and N. Songsasen. 2021. Canid reproductive biology: norm and unique aspects in strategies and mechanisms. Animals, 11 (3): 653.

Netherton, J. K., B. R. Robinson, R. A. Ogle, A. Gunn, A. I. S. B. Villaverde, K. Colyvas, C. Wise, T. Russo, A. Dowdell and M. A. Baker. 2022. Seasonal variation in bull semen quality demonstrates there are heat-sensitive and heat-tolerant bulls. Scientific Reports, 12 (1): 15322.

Prapaiwan, N., T. Tharasanit, S. Punjachaipornpol, D. Yamtang, A. Roongsitthichai, W. Moonarmart, K. Kaeoket and S. Manee-In. 2016. Low-density lipoprotein improves motility and plasma membrane integrity of cryopreserved canine epididymal spermatozoa. Asian-Australasian Journal of Animal Sciences, 29 (5): 646.

Priyanto, L., S. Herdis, P. I. Sitaresmi, T. P. Priyatno, R. I. Anwar, D. A. Mahari, F. B. I. Lupitasari, M. Surachman, I. W. A. Darmawan and A. Abrar. 2023. The effects of various high dosage of α-Tocopherol and ascorbic acid in Tris egg yolk extender on post-thawed sperm quality in tropical Brahman bulls. Pakistan Journal of Zoology, pp.1-9.

Qazi, I.H., C. Angel, H. Yang, E. Zoidis, B. Pan, Z. Wu, Z. Ming, C. J. Zeng, Q. Meng and H. Han. 2019. Role of selenium and selenoproteins in male reproductive function: a review of past and present evidences. Antioxidants, 8 (8): 268.

Sandeep, S., P. Bamel, M. V. Meenakshi Virmani, R. Malik and G. S. Gyan Singh. 2015. Effect of vitamin E on the seminal and biochemical parameters of Murrah buffalo bull semen during different stages of freezing, 16 (1): 33-35.

Sicherle, C. C., F. F. D. Souza, C. B. P. Freitas-Dell'Aqua, G. B. Mothé, C. R. Padovani, F. O. Papa and M. D. Lopes. 2020. Effects of the cryopreservation process on dog sperm integrity. Animal Reproduction, 17 (1): e20190081.

Silva, S., A. Soares, A. Batista, F. Almeida, J. Nunes, C. Peixoto and M. Guerra. 2011. In vitro and in vivo evaluation of ram sperm frozen in tris egg-yolk and supplemented with superoxide dismutase and reduced glutathione. Reproduction in Domestic Animals, 46 (5): 874-881.

Suharyati, S., M. Hartono, S. Siswanto, E. Febriyansar and M. M. P. Sirat. 2024. The Effect of Different Types of Egg Yolk in Tris Diluent on the Quality of Frozen Semen of

Brahman Cattle. Jurnal Ilmiah Peternakan Terpadu, 12 (2): 122-141.

Zmudzinska, A., M. A. Bromke, R. Strzezek, M. Zielinska, B. Olejnik and M. Mogielnicka-Brzozowska. 2022. Proteomic Analysis of Intracellular and Membrane-Associated Fractions of Canine (Canis lupus familiaris) Epididymal Spermatozoa and Sperm Structure Separation. Animals, 12 (6): 772.

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