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# Post Thawed Quality of Tharparkar Bull Semen Extended in Bioxcell™ and Tris-Based Egg Yolk Extenders Supplemented with Vitamin E (α-Tocopherol)

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## ABSTRACT

The study was designed to determine the effect of Vitamin E (α-tocopherol) supplimentation into BIOXcell™ and Tris based egg yolk extender on post-thawed quality of Tharparkar cattle bull semen. The research was carried out at Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agricultural University Tandojam. In this study four fertile Tharparkar bulls (A, B, C and D) having age of 4-5 years were used. A total of 52 (n=13) ejaculates were collected with the help of artificial vagina, after the collection macroscopic (color, volume and pH) and microscopic (motility, morphology, viability, concentration and membrane integrity) parameters were observed. The samples having motility, morphology, viability and membrane integrity ≥70% were pooled and processed. Pooled semen samples were divided into four groups and diluted with Tris, Tris+Vitamin E(α-tocopherol) and BIOXcell™, BIOXcell™+Vitamin E (α-tocopherol). Post-thawed assessment of motility, morphology, viability and membrane integrity with BIOXcell™+ Vitamin E(α-tocopherol) (58.31±0.86, 76.22±1.04, 71.27±1.44, 64.68±1.43) showed improved quality parameters as compared to Tris+ Vitamin E(α-Tocopherol) (48.68±0.68, 64.45±1.54, 66.22±1.75, 58.25±1.08), BIOXcell™ (45.72±0.53, 67.45±0.84, 64.72±1.67, 56.63±1.39) and Tris (43.54±0.49, 64.68±0.80, 65.18±1.33, 54.41±1.36). On the basis of in vitro results BIOXcell™+Vitamin E(α-tocopherol) showed improved post-thawed quality parameters and it was used for artificial insemination. A total of 20 animals were synchronized at Bhens colony; Ghumanrabad with one injection prostaglandin (PGF2α) protocol and 13 animals showed estrus and were inseminated. Meanwhile, 8 were found pregnant through rectal palpation with a pregnancy rate of 61.53%.

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**AC and AK conceptualized and leaded original manuscript writeup. AAM guided and helped in revision of research article. MB, QK, SAT and MIP helped in the writeup of material and method.**

**Key words**
**Tharparkar bull semen, Reactive oxygen species, Cryopreservation, Vitamin E (α-tocopherol), Thawing, Post thaw**

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## INTRODUCTION

Livestock plays a pivotal role in the social and economic development of Pakistan. Livestock is the most important sector of agriculture. It contributes up to 60.1 % in the agricultural value added and about 11.7 % to the gross domestic product (GDP) of Pakistan during 2020-21, Moreover total livestock population in Pakistan

is 213.1 million heads including cattle 51.5 million (GOP, 2020-21). Cattle belong to the family Bovidae. Cattle were originated from *Bostaurus* or humpless cattle (Europe) and *Bosindicus* or humped cattle (Indo-Pakistan), The cattle breeds of Pakistan are Red Sindhi, Bhagnari, Sahiwal, Dhanni, Kankrej, Rojhan, Lohani, Cholistani, Dajal and Tharparkar (Farooq *et al*., 2013).Tharparkar is a lyre-horned breed that originated from the Thar Desert (Chand, 2011; Memon *et al*., 2022). It is one of the main breeds which is found in the Thar Desert mainly located in the India-Pakistan border,It is considered a dual purpose breed utilized as a draught and milking animal (Godara *et al*., 2015). Meanwhile, it has been blessed with the capabilities of heat tolerant, tick resistant, disease-resistant and a stable aboriginal indigenous breed.Tharparkar breed is producing approximately 5-10 L of milk per day and round about 1135-2000 L of milk per lactation (Choudhary *et al*., 2018).

Extenders are used for conservation, preservation and extension of semen. Moreover, extenders are also used to prevent different types of damages while processing, storage and shipping of semen. During the last 40 years, different extenders have been used for the evaluation of spermatozoa during cooling and post-thawing. Egg yolk-based semen diluents are readily used for the cryopreservation of semen (Apu *et al*., 2012; Emamverdi *et al*., 2014). Different components are combined for the preparation of semen extenders, such that they own all properties which have capabilities to protect the life of spermatozoa during extension at ambient environment and cryopreservation. It must be isotonic (maintain osmotic pressure) (280-310 mOsm/kg), maintain pH, cold shock defense, act as a source of energy (spermatozoa metabolism), antimicrobial, protect during cooled and post thawing and able to preserve sperm fertility for the long time duration (Raheja *et al*., 2018). Tris-based egg yolk extender is used for the extension of semen though it is a mixture of a substance having cryoprotectant properties. With long time storage, it shows qualities of a great stabilizing agent with constant results (Apu *et al*., 2012; Emamverdi *et al*., 2014).

BIOXcell™ is a commercial lecithin based extender. However, it has been used in many studies on exotic breeds but in Tharparkar cattle it's merely not used. Moreover, it has positive effects on the cryopreservation of semen so there was a dire need to study the effect of BIOXcell™ extender in Tharparkar cattle bull semen. BIOXcell™ has merits in case of lower sanitary risks, its chemically defined and ready to use meanwhile its commercially available (Akhter *et al*., 2010). The process of cryopreservations also leads to various intracellular changes which results in the production of reactive oxygen species (ROS) that are indirect cause of reduced reproductive capacity, DNA damage, increased membrane permeability. ROS damages plasma membranes and DNA molecules in the sperm and other cells. High levels of superoxide ions, peroxynitrates hydrogen peroxide, harm the components of cells such as membrane lipids, organelles, proteins and DNA. To overcome ROS antioxidants are added, Vitamin E is the main antioxidant sited within the biological membranes that perform a key role in defending from lipid peroxidation. α-tocopherol breaks the chain reactions of lipid peroxidation but the mechanism of donation of a hydrogen atom from its phenolic hydroxyl group to lipid peroxyl radical resulting in the creation of stable lipid hydroperoxide and unreactive tocopheroxyl radicals. The antioxidant property of vitamin E can improve the post-thawed traits of spermatozoa and could improve male fertility. Vitamin E has the antioxidant property that supports sperm against ROS damage (Almbro *et al*., 2011). The addition of antioxidant agents in semen extenders improves sperm longevity, individual motility, progressive motility and viability (Aminipour *et al*., 2013). Though cattle bull semen has a natural defense system against the ROS but that is not enough to prevent sperm against changes in decrease and increase in the temperature during the process of cryopreservation (Nichi *et al*., 2006; Khan *et al*., 2021). Artificial insemination and estrus synchronization are the techniques that provide opportunities to control the estrus cycle and reproductive management in bovines (Senger, 2005; Lijalem *et al*., 2015). For estrus synchronization, it is necessary to have cyclic cows with proper nutrition, good body condition scores (BCS) and high-quality semen (Lamb, 2012; Gizaw and Dima, 2016). There were several studies available on crypreservation but few were reported on indigenous Tharparkar cattle bull semen. Therefore, this study was designed with a hypothesis that supplementation of vitamin E (α-tocopherol) into BIOXcell™ and Tris-based egg yolk extender would enhance the post-thawed quality of Tharparkar cattle bull semen.

## MATERIALS AND METHODS

The study was carried out at Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agricultural University Tandojam. In this study four fertile Tharparkar bulls (A, B, C and D) having age of 4-5 years were used. A total of 52 (n=13) ejaculates were collected with the help of artificial vagina, After the collection macroscopic (color, volume and pH) and microscopic (motility, morphology, viability, concentration and membrane integrity) parameters were observed. The samples having motility, morphology, viability and membrane integrity ≥ 70% were pooled and processed. Pooled semen samples were divided into four groups and diluted with Tris, Tris +Vitamin E(α-tocopherol) 0.02 Mm/ml and BIOXcell™, BIOXcell™+ Vitamin E(α-tocopherol) 0.02 Mm/ml.

The volume of semen was observed through visual examination with the help of a graduated tube. Visual examination was done for judgment of colour however semen was categorized as milky, creamy white and translucent (Kaka *et al*., 2012). pH of semen was determined by using a digital pH meter (Hanna Company, Instrument number #HI98107, software version v1.01, serial number # HA02122313, Made in Romania).

The wave motion was assessed on a clean warm dry slide by putting a drop of undiluted semen under low power magnification (10X) with a phase-contrast microscope (Nikon, Germany). The wave sample was recorded and classified as described by Rehman (2012).

For the assessment of motility, the semen sample was diluted in normal saline at 1:100. After one drop of diluted sample was taken and put on a pre-warmed slide by applying a cover slip on it. While using (20 xs) underlow magnification Motility was observed. Motility percentage was taken by selecting 100 spermatozoa moving randomly in a straight forward direction. Spermatozoa that were stirring in a backward direction or circle were not counted moreover results were expressed in motility percentage. The sample with 70% motility was processed for further assessment.

The concentration of sperm was assessed with the help of haemocytometer as described by Kaka *et al*. (2012). As per the standard staining procedure of sperm, morphology was determined as described by (Kaka *et al*., 2012) and The viability of sperms was recorded as described by Kaka *et al*. (2012). Hypo osmotic swelling (HOST) test method was used to determine the membrane integrity of fresh semen samples as described by (Kaka *et al*., 2012).

Each semen sample was diluted with Tris-based egg yolk extender Tris 3.81g, Citric acid 1.97g, D (-) fructose 1.25g, egg yolk 20ml, Glycerol 7ml, Pencillin 1000 IU, Streptomycin 1.00 g, Distilled water 100, and BIOXcell™ (IMV technologies, L' Agile, France) as described by Kaka (2015), Tris 2.3 g, Sodium citrate 6.2 g, Potassium chloride 0.8 g, Fructose 1.2 g, Monohydrate lactose 0.8 g, Glycine 0.2 g, Anhydrous glucose 0.5 g, Taurine 0.005 g, Gentamicin sulphate 0.24 g, Tylosin tartrate 0.33 g, Linco-spectin 100 0.383 g, Glycerol 40.2 g, Hydrate of calcium lactate 0.7 g, Soy lecithin 1.5 g, Monohydrate citric acid 2.5 g, Ultrapure water 1000 g (Penitente-Filho *et al*., 2014).

The diluted semen were packed into straws (0.25 ml). Moreover, straws were classified as red for BIOXcell™ (control group), white for BIOXcell™(vitamin 0.2mM), yellow for tris-based egg yolk extender (control group) and blue for tris-based egg yolk extender (vitamin 0.2mM). Semen straws were filled with a manual suction machine. The semen straws were sealed with polyvinyl pyrrolidone powder (PVP). The process of filling and sealing were performed in the cold cabinet to maintain a similar temperature and prevent shock. The equilibration period was completed within 2 h at 5°C.

Freezing was carried out by holding the straws in liquid nitrogen vapors 5 cm above the surface of liquid nitrogen for 6 min as described by Kaka *et al*. (2012). Then the straws were plunged into liquid nitrogen (-196 °C). The frozen semen were stored in liquid nitrogen at least for 24 h and then assessed for post-thaw qualities parameters i.e. motility, morphology, viability and membrane integrity were followed (Rasul *et al*., 2000; Kaka *et al*., 2012).

The collected data were subsequently subjected to a one-way analysis of variance (ANOVA) using Statistics (2006) and LSD was used to determine the difference among means of different groups.

## RESULTS AND DISCUSSION

The results of macroscopic parameters i.e. volume, color, pH and microscopic parameters i.e. wave motion, motility, concentration, morphology, viability and membrane integrity of freshly collected semen from bulls A, B, C and D are depicted in Table I. Results showed a slightly highest (Mean % ± SEM) values of semen from bull D. However, no significant difference (P>0.05) were recorded amongst bulls.

**Table I. Characteristics of fresh Tharparkar bull semen (Mean % ± SEM).**

| Parameters | Bull A | Bull B | Bull C | Bull D |
|---|---|---|---|---|
| Volume | 6.46±0.17$^{a}$ | 6.29±0.15$^{a}$ | 6.24±0.16$^{a}$ | 6.50±0.19$^{a}$ |
| Color | Creamy white | Creamy white | Creamy white | Creamy white |
| pH | 6.59±0.04$^{a}$ | 6.56±0.04$^{a}$ | 6.58±0.04$^{a}$ | 6.61±0.04$^{a}$ |
| Wave motion | ++++ | ++++ | ++++ | ++++ |
| Motility | 89.81±0.88$^{ab}$ | 87.27±1.07$^{b}$ | 88.09±1.56$^{ab}$ | 90.63±0.56$^{a}$ |
| Concentration (×10$^{6}$/ml) | 1190.5±9.47$^{a}$ | 1180.9±18.38$^{a}$ | 1186.8±15.81$^{a}$ | 1203.6±21.08$^{a}$ |
| Morphology | 90.45±0.56$^{a}$ | 90.09±0.56$^{a}$ | 91.09±0.56$^{a}$ | 91.18±0.81$^{a}$ |
| Viability | 88.09±0.39$^{a}$ | 87.09±0.62$^{ab}$ | 86.36±0.69$^{b}$ | 88.27±0.38$^{a}$ |
| Membrane integrity | 85.45±0.97$^{a}$ | 84.27±0.82$^{a}$ | 85.63±0.78$^{a}$ | 87.09±1.31$^{a}$ |

a, ab: Values with different superscripts within columns shows significant difference

**Table II. Post-thaw assessment of Tharparkar bull semen (Mean % ± SEM) in BIOXcell™ and Tris based egg yolk extender supplemented with and without vitamin E (α-tocopherol).**

| Extender | Motility | Morphology | Viability | Membrane integrity |
|---|---|---|---|---|
| Tris+Control | 43.54±0.49$^{d}$ | 64.68±0.80$^{b}$ | 65.18±1.33$^{b}$ | 54.41±1.36$^{c}$ |
| Tris+vitamin E (α-tocopherol) 0.02 mM/ml | 48.68±0.68$^{b}$ | 64.45±1.54$^{b}$ | 66.22±1.75$^{b}$ | 58.25±1.08$^{b}$ |
| BioXcell™-Control | 45.72±0.53$^{c}$ | 67.45±0.84$^{b}$ | 64.72±1.67$^{b}$ | 56.63±1.39$^{bc}$ |
| BioXcell™+ vitamin E (α-tocopherol) 0.02 mM/ml | 58.31±0.86$^{a}$ | 76.22±1.04$^{a}$ | 71.27±1.44$^{a}$ | 64.68±1.43$^{a}$ |

a, b, c, d value with different superscripts within columns shows significant difference (P<0.05).

Table II represent the (Mean±SEM) post-thaw assessment of semen quality parameters i.e. motility, morphology, viability and membrane integrity in BIOXcell-™ and tris based egg yolk extender supplemented with and without vitamin E (α-tocopherol). Improved quality parameters were observed in BIOXcell™ supplemented with vitamin E (α-tocopherol). Moreover, significant difference (P<0.05) was observed among all groups.

Table III presents the percentage of conception rate obtained from cows synchronized through one shot prostaglandin and inseminated with Tharparkar bull semen diluted with BIOXcell™ (supplemented with 0.02mm of vitamin E (α-tocopherol).

**Table III. Conception rate of cows inseminated with Tharparkar bull semen diluted with BIOXcell™ (supplemented with 0.02mm of vitamin E (α-tocopherol).**

| Cows | 20 |
|---|---|
| Synchronized | 20 |
| Animals came in heat | 13 |
| Inseminated | 13 |
| Animals got conceived | 8 |
| Conception rate (%) | 61.53 |

Post-thaw motility of Tharparkar bull was observed in BIOXcell™ and Tris based egg yolk extender supplemented with and without vitamin E (α-tocopherol) 0.02 Mm/ml. The spermatozoa showed improved post-thaw results in BIOXcell™ supplemented with vitamin E (α-tocopherol) 0.02 Mm/ml, which ranged up to 58.31±0.86 and the results of motility of the current study agreed with the results obtained by Towhidi and Parks (2012), Kaka (2015) and Yadav *et al*. (2019). Their observed means were 61.67±0.59, 51.50±0.6 and 60.08±0.64. However, the value was lower than the findings observed by Motemani *et al*. (2017) 64.1±1.6. Meanwhile higher than the observations of Towhidi and Parks (2012), Ansari *et al*. (2012), and Kaka *et al*. (2015a, b, 2016), their recorded mean of findings was as 41.4±0.4, 48.54±1.6, 45.5±0.2, 48.94±0.83 and 48±1.0. The fluctuation in results might be due to the use of antioxidants at various concentrations and processing techniques. However, Towhidi and Parks (2012); Kaka (2015), Kaka *et al*. (2015b, 2016) and Yadav *et al*. (2019) studied motility in BIOXcell™ extender at various concentrations of vitamin E (α-tocopherol).

Post-thaw morphology of Tharparkar bull semen was also greater in the group of BIOXcell™ supplemented with vitamin E (α-tocopherol) 0.02 Mm/ml. Which obtained results ranging from 76.22±1.04. Current findings agreed with Ansari *et al*. (2012) and Motemani *et al*. (2017), 75±3.1 and 75±1.7. However, the value means the value of the present study is higher than the results carried out by Kaka (2015), and Kaka *et al*. (2015a, b, 2016). Their results were 71.00±1.7, 72±1.2, 70.63±0.54 and 66.25±3.4. The difference in results may be due to processing techniques, the use of extenders with different compositions, or it depends on the use of antioxidants. Meanwhile, the use of various concentrations of antioxidants presents variations in results. Meanwhile, Ansari *et al*. (2012) and Motemani *et al*. (2017) studied morphology in BIOXcell™ extender at various concentrations of vitamin E (α-tocopherol).

The highest viability of the current study was also obtained in BIOXcell™ supplemented with vitamin E (α-tocopherol) 0.02 Mm/ml. The obtained result was 71.27±1.44. Meanwhile, the result agreed with studies of Kaka (2015), and Kaka *et al*. (2015a, b, 2016, 2019). Their recorded values were as 71.75±1.5, 74±1.4, 73.42±0.96, 69.75±2.7 and 69.7±0.33. Value was higher than findings obtained by Towhidi and Parks (2012), Ansari *et al*. (2012) and Motemani *et al*. (2017), 45.1±1.4, 61.70±0.25 and 64.1±1.8. The variations in findings may be due use of antioxidants at various concentrations, processing techniques and so on so forth.

In the last membrane integrity was also highest in group BIOXcell™ supplemented with vitamin E (α-tocopherol) 0.02 mM/ml. The findings were 64.68±1.43. The result of the present study agreed with the results of Motemani *et al*. (2017), 61.7±1.6 and lower than Kaka (2015), and Kaka *et al*. (2015a, b), 70.00±2.9, 75±1.6 and 74.84±1.8, respectively. Meanwhile, the value was higher

than Ansari *et al*. (2012), 60.11±2.3. These variations in research findings may be due to variance of management, use of antioxidants at various concentrations and another handling/ processing factors (Sansone *et al*., 2000).

## CONCLUSION

On the basis of the current study, it concluded that BIOXcell™ and Tris-based egg yolk extender supplement with vitamin E (α-tocopherol) showed improved post-thawed quality parameters of Tharparkar bull semen.

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*IRB approval*

The work was approved by the departmental board of studies (BOS), Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences Sindh Agriculture University on 18th November 2021.

*Ethics statement*

All procedures in this study were conducted in accordance with approved ethical policies and protocols of SPU and Animal Reproduction Farm under Department of Animal Reproduction, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tanddojam.

*Statement of conflict of interest*

The authors have declared no conflict of interest.

## REFERENCES

Akhter, S., Ansari, M.S., Rakha, B.A., Andrabi, S.M.H., Iqbal, S. and Ullah, N., 2010. Cryopreservation of buffalo (*Bubalus bubalis*) semen in BIOXcell® extender. *Theriogenology*, **74**: 951-955. https://doi.org/10.1016/j.theriogenology.2010.04.024

Almbro, M., Damian, K. and Leigh, W., 2011. Effects of vitamin E and beta-carotene on sperm competitiveness. *Ecology*, **14**: 891–895. https://doi.org/10.1111/j.1461-0248.2011.01653.x

Aminipour, H., Tahmasbiand, A.M. and Naserain, A.A., 2013. The influence of vitamin E on semen characteristics of Ghezel rams in during cooling and frozen process. *Eur. Zool. J.,* **2**: 94–99.

Ansari, M., Towhidi, A., Shahrbabak, M.M. and Bahreini, M., 2012. Docosahexaenoic acid and alpha-tocopherol improve sperm cryosurvival in goat. *Sci. J. agric. Sci.*, **45**: 7-13.

Apu, A.S., Yahia, K.M., Hussain, S.S., Fakruzzaman, M. and Notter, D.R., 2012. A comparative study of fresh and frozen-thawed semen quality in relation to fertility of black bengal goats semen from six adult male black bengal goats. *Iran. J. appl. Anim. Sci*., **2**: 157-161.

Chand, T., 2011. *Genetic evaluation of lifetime productivity in Tharparkar cattle.* Doctoral dissertation, Rajasthan University of Veterinary and Animal Sciences, Bikaner-334001.

Choudhary, S., Yamini, Barman, D., Prasad, K.C., Panchbhai, G., Kumar, R. and Kumar, A., 2018. Tharparkar: The pride of desert. *J. Ent. Zool. Stud*., **6**: 1915-1919.

Emamverdi, M., Zhandi, M., Shahneh, A.Z., Sharafi, M., Akhlaghi, A., Motlagh, M.K., Dadkhah, F. and Davachi, N.D., 2014. Flow cytometric and microscopic evaluation of post-thawed ram semen cryopreserved in chemically defined home-made or commercial extenders. *Anim. Prod. Sci*., **55**: 551-558. https://doi.org/10.1071/AN13215

Farooq, U., Ijaz, A., Ahmad, N., Rehman, H. and Zaneb, H., 2013. Investigations on semen quality and freezability of Cholistani breeding bulls-A preliminary study from Cholistan desert of Pakistan. *J. appl. Pharm. Sci*., **23**: 359-363.

Gizaw, T. and Dima, F.G., 2016. Assessment of problems and constraints associated with artificial insemination in the two selected districts of Arsi Zone. *J. Hlth. Res*., **3**: 14–37.

Godara, A.S., Tomar, A.K.S., Patel, M., Godara, R.S., Bhat, S.A. and Bharati, P., 2015. Body conformation in Tharparkar cattle as a tool of selection. *J. Anim. Res*., **5**: 423-430. https://doi.org/10.5958/2277-940X.2015.00073.X

GOP, 2020-21. *Pakistan economic survey, Government of Pakistan*. Finance Division, Economic Advisor Wing, Islamabad, pp. 35-37.

Kaka, A., Samo, M.U., Rahoo, T.H., Rehman, Z.U., Shah, Z., Mushtaq, M., Kaka, U. and Behan, A.A.,

2012. Study on post thawing quality of Kundhi buffalo semen. *J. app. Pharm. Sci.*, **22(2 Supp.)**: 59-62.

Kaka, A., 2015. http://psasir.upm.edu.my/id/eprint/56730/1/FPV%2012RR, pp. 88-118.

Kaka, A., Samo, M.U., Rahoo, T.H., Rehman, Z.U., Shah, Z., Mushtaq, M., Kaka, U. and Behan, A.A., 2015a. Study on post-thawing quality of Thari semen. *J. appl. Pharm. Sci.,* **22**: 59-62.

Kaka, A., Wahid, H., Rosnina, Y., Yimer, N., Khumran, A.M., Sarsaifi, K., Behan, A.A., Kaka, U. and Ebrahimi, M., 2015b. α-Linolenic acid supplementation in BioXcell® extender can improve the quality of post-cooling and frozen-thawed bovine sperm. *Anim. Reprod. Sci*., **153**: 1-7. https://doi.org/10.1016/j.anireprosci.2014.12.001

Kaka, A., Memon, A.A., Khatri, P., Kumbhar, H.K., Kalhoro, D.H., Tariq, M., Pirzado, S.A. and Tunio, A.N., 2016. Determination of quality characteristics of Kundhi buffalo bull semen. *J. Basic appl. Sci*., **12**: 394-397. https://doi.org/10.6000/1927-5129.2016.12.61

Khan, I.M., Xu, D., Cao, Z., Liu, H., Khan, A., Rahman, S.U., Ahmad, J.Z., Raheem, M.A. and Zhang, Y., 2021. Addition of l-cysteine and vitamin e to semen diluent enhances freeze-thawed spermatozoa characteristics in crossbred cattle bulls under subtropical environment. *Pakistan J. Zool.*, **53**: 1309-13196. https://doi.org/10.17582/journal.pjz/20191006091046

Lamb, G.C., 2012. *Control of estrus in cows*. Proceedings of the applied reproductive strategies in beef cattle, pp. 85-97. **Author: Please check this reference**

Lijalem, T., Abebe, M. and Haile, B., 2015. Breeding technology assessment at small holder dairy cattle production level in selected districts of hadya zone, Southern Ethiopia. *J. Adv. Life Sci.*, **34**.

Memon, A.S., Kaka, A., Laghari, I.H., Channo, A., Bakhsh, M., Kalwar, Q. and Sethar, A., 2022. Effect of soybean milk and tris egg yolk based extender on semen quality parameters of tharparkar bull. *Univ. Sindh J. Anim. Sci*., **6**: 20-25.

Motemani, M., Chamani, M., Sharafi, M. and Masoudi, R., 2017. Alpha-tocopherol improves frozen-thawed sperm quality by reducing hydrogen peroxide during cryopreservation of bull semen. *Spanish J. agric. Res.*, **15**: 15. https://doi.org/10.5424/sjar/2017151-9761

Nichi, M., Bols, P.E.G., Zuge, R.M., Barnabe, V.H., Goovaerts, I.G.F., Barnabe, R.C. and Cortada, C.N.M., 2006. Seasonal variation in semen quality in Bosindicus and Bostaurus bulls raised under tropical conditions. *Theriogenology*, **66**: 822–828. https://doi.org/10.1016/j.theriogenology.2006.01.056

Penitente-Filho, J.M., Oliveira, F.A., Jimenez, C.R., Carrascal, E., Dias, J.C.O., Oliveira, G.D., Silveira, C.O. and Torres, C.A.A., 2014. Association of vitamin E with rapid thawing on goat semen. *Sci. World J.*, **2014**. https://doi.org/10.1155/2014/964172

Raheja, N., Choudhary, S., Grewal, S., Sharma, N. and Kumar, N., 2018. A review on semen extenders and additives used in cattle and buffalo bull semen preservation. *J. Ent. Zool. Stud*., **6**: 239-245.

Rasul, Z., Anzar, M., Jalali, S. and Ahmad, N., 2000. Effect of buffering systems on post-thaw motion characteristics, plasma membrane integrity, and acrosome morphology of buffalo spermatozoa. *Anim. Reprod. Sci*., **59**: 31-41. https://doi.org/10.1016/S0378-4320(00)00070-1

Rehman, F.U., 2012. *Substitution of animal protein source with plant protein in semen extenders of various cattle breeds*. Doctoral dissertation, M.Phil. Tesis, Faculty of Animal Husbandry and Veterinary Sciences Khyber Phaktunkhwa Agricultural University Peshawar Pakistan. Pakistan. pp. 9-20.

Sansone, G.M.J.F., Nastri, M.J.F. and Fabbrocini, A., 2000. Storage of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci*., **62**: 55-76. https://doi.org/10.1016/S0378-4320(00)00154-8

Senger, P.L., 2005. *Pathway to pregnancy and parturition* 2nd revised ed., Current Conceptions Inc. pp. 144–188.

Towhidi, A. and Parks, J.E., 2012. Effect of n-3 fatty acids and α-tocopherol on post-thaw parameters and fatty acid composition of bovine sperm. *J. Assist. Reprod. Genet*., **29**: 1051-1056. https://doi.org/10.1007/s10815-012-9834-7

Yadav, D., Singh, V., Yadav, S.S., Patel, A., Kumar, A., Sonkar, V., Kumar, A., Yadav, S., Kumar, B., Yadav, B. and Saxena, A., 2019. Effect of glutathione on viability and progressive motility of Hariana bull spermatozoa during cryopreservation in semi-arid region. *J. Pendidik. Indonesia*, **8**: 871-876.